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PATENT
Docket No.: ALXN-P01-013

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICATION NUMBER:	08/487,283
PATENT NUMBER:	6,355,245
FILING DATE:	June 7, 1995
ISSUE DATE:	March 12, 2002
INVENTORS:	Evans, et al.
ASSIGNEE:	Alexion Pharmaceuticals, Inc.
TITLE:	ANTIBODIES TO HUMAN COMPLEMENT COMPONENT C5 (as shown on Certificate of Correction)

MS Patent Ext.
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPLICATION FOR EXTENSION OF PATENT TERM UNDER 35 U.S.C. §156 AND 37 CFR §1.740

Sir:

Applicant, Alexion Pharmaceuticals, Inc., 352 Knotter Drive, Cheshire, CT 06410, hereby files this application under 35 U.S.C. §156 and 37 C.F.R. §1.740 for extension of term of U.S. Patent No. 6,355,245, issued March 12, 2002 based on an application filed June 7, 1995, which claims the benefit of priority under 35 U.S.C. §120 to international application No. PCT/US95/05688, filed May 1, 1995, and U.S. patent application U.S. Serial No. 08/236,208, filed May 2, 1994, now U.S. Patent No. 6,074,642. The current expiration date of this patent is seventeen years from the issue date, or March 12, 2019. The extension request is for a period of 735 days to March 16, 2021, which is fourteen years from the BLA approval date.

In accordance with the provisions of 37 C.F.R. §1.740, Applicants provide the following information:

1. Identification of the Approved Product (37 C.F.R. §1.740(a)(1))

On March 16, 2007, the U.S. Food and Drug Administration (FDA) approved Soliris™ a formulation of eculizumab for the treatment of patients with paroxysmal nocturnal hemoglobinuria (PNH) to reduce hemolysis.

Eculizumab is a recombinant humanized monoclonal $IgG_{2/4}\kappa$ antibody that specifically binds to the complement protein C5 and inhibits its cleavage to C5a and C5b. Eculizumab contains human constant regions from IgG sequences and murine complementarity-determining regions (CDRs) grafted onto the human framework light- and heavy-chain variable regions. Eculizumab is composed of two 448 amino acid heavy chains and two 214 amino acid light chains and has a molecular weight of approximately 148 kDa. Eculizumab is produced in mammalian (murine myeloma) cell culture.

Soliris™ is a sterile, clear, colorless, preservative-free 10 mg/mL solution for intravenous infusion and is supplied in 30-mL single-use vials. Soliris™ is formulated at pH 7 and each vial contains 300 mg of eculizumab, 13.8 mg sodium phosphate monobasic, 53.4 mg sodium phosphate dibasic, 263.1 mg sodium chloride, 6.6 mg polysorbate 80 (vegetable origin) and Water for Injection, USP. A copy of the package insert is attached hereto as Exhibit A.

2. Identification of Federal Statute Under Which Regulatory Review Occurred (37 C.F.R. §1.740(a)(2))

The approval for Soliris™ was granted by the Food and Drug Administration (FDA) pursuant to 42 U.S.C. §262, The Public Health and Welfare Act.

3. Identification of Date on Which Approved Product Received Permission for Commercial Marketing or Use (37 C.F.R. §1.740(a)(3))

The Approved Product received permission for commercial marketing or use in a letter dated March 16, 2007, from Richard Pazdur, M.D., Director, Office of Oncology Drug Products, Center for Drug Evaluation and Research, U.S. Food and Drug Administration. A redacted copy of the approval letter is attached hereto as Exhibit B.

4. Identification of Active Ingredient (37 C.F.R. §1.740(a)(4))

The sole active ingredient of the approved new drug is eculizumab as identified above under Section 1. Eculizumab has not been previously approved for commercial marketing or use under the Federal Food, Drug, and Cosmetic Act (FFDCA), the Public Health Service Act, or the Virus-Serum-Toxin Act.

5. Timely Filing of This Application (37 C.F.R. §1.740(a)(5))

This application is filed, pursuant to 35 U.S.C. §156(d)(1) and 37 C.F.R. §1.720(f), within the permitted sixty-day (60-day) period that began on March 16, 2007, the approval date for Soliris™, and that will expire on May 15, 2007.

6. Identification of the Patent for Which an Extension Is Sought (37 C.F.R. §1.740(a)(6))

Inventors:	Mark J. Evans, Louis A. Matis, Eileen Elliott Mueller, Steven H. Nye, Scott Rollins, Russell P. Rother, Jeremy P. Springhorn, Stephen P. Squinto, Thomas C. Thomas, James A. Wilkins
Patent No.:	6,355,245
Issued:	March 12, 2002
Expiration:	March 12, 2019

7. Copy of Patent Attached (37 C.F.R. §1.740(a)(7))

A copy of U.S. Patent No. 6,355,245, for which an extension is being sought, is attached in its entirety as Exhibit C.

8. Disclaimers, Certificates of Correction, Receipts of Maintenance Fee Payment or Reexamination Certificate (37 C.F.R. §1.740(a)(8))

A copy of the Maintenance Fee Statement showing payment of year 4 fees is attached as Exhibit D.

A copy of the Certificate of Correction issued in association with U.S. 6,355,245 is attached as Exhibit E.

The patent for which extension is being sought has not been the subject of any disclaimer or reexamination certificate.

9. Statement of Patent Claim Coverage of Approved Product (37 C.F.R. §1.740(a)(9))

U.S. Patent No. 6,355,245 claims the Approved Product. The applicable patent claims are 1-7, 9, 10, 12-15, 17, 19 and 23. The manner in which representative claims read on the Approved Product is set forth in the chart below.

Applicable Claims of U.S. Patent No. 6,355,245	Manner in Which Each Claim Reads on Approved Product or Method of Using the Approved Product
1. An antibody comprising at least one antibody-antigen binding site, said antibody exhibiting specific binding to human complement component C5, said specific binding being targeted to the alpha chain of human complement component C5, wherein the antibody 1) inhibits complement activation in a human body fluid, 2) inhibits the binding of purified human complement component C5 to either human complement component C3 or human complement component C4, and 3) does not specifically bind to the human complement activation product free C5a.	Eculizumab is an antibody which binds to human complement C5 (see Hill et al., Blood 106:2559-2565 (2005) (Exhibit F) (see, e.g., Abstract). Also see Thomas et al, Mol. Immunol. 33:1389-1401 (1996) (Exhibit H). Parent murine antibody 5G1.1 binds the alpha chain of C5 (see column 38, lines 52-67 of the '245 patent).[1] A phase 3 clinical trial showed that eculizumab inhibits complement activation in humans (see Hillmen et al., N. Engl. J. Med. 355:1233-1243 (2006) (Exhibit G) (see, e.g., Background section of the Abstract); Hillmen et al. N. Engl. J. Med. 350:552-559 (2004) (Exhibit L); and Hill et al. Blood 106:2559-2565 (2005) (Exhibit F)). 5G1.1 was shown to inhibit binding of C5 to C3 and C4 (see Example 14 at column 53 of the '245 patent). 5G1.1 was found not to bind to free C5a (see Example 13 of the '245 patent, especially column 51, lines 39-56).
2. The antibody of claim 1 wherein the inhibition of complement activation in the human body fluid is measurable as an increment of blockade of C5a generation and an increment of blockade of complement hemolytic activity in the body fluid, said increment of blockade of C5a generation being substantially equal to said increment of blockade of complement hemolytic activity.	Inhibition of C5a generation in 40% human serum was shown using a humanized 5G1.1 Fab fragment and a humanized single-chain antibody version of 5G1.1, e.g., the Fab fragment and a single-chain version of eculizumab (see Thomas et al., Mol. Immunol. 33:1389-1401 (1996)) (Exhibit H) (see first paragraph on page 1396 and Figure 8). Inhibition of complement hemolytic activity in 40% human serum was shown using the same Fab fragment and single-chain versions of eculizumab (Exhibit H) (see final paragraph on

[1] The CDRs of eculizumab are from the parent murine 5G1.1 antibody. Humanization of an antibody does not alter its binding specificity and humanization of eculizumab did not significantly affect binding affinity.

Applicable Claims of U.S. Patent No. 6,355,245	Manner in Which Each Claim Reads on Approved Product or Method of Using the Approved Product
	page 1395 and Figure 6B). The concentration of inhibitor required for C5a generation (Figure 8) was comparable to the concentration of inhibitor required for inhibition of lysis (Figure 6B) (quotation from page 1396 of Exhibit H).
6. The antibody of claim 1 wherein the inhibition of complement activation in the human body fluid is measurable as a substantially complete blockade of C5a generation in the body fluid and a substantially complete blockade of complement hemolytic activity in the body fluid when the antibody is added to the body fluid at a concentration yielding a ratio equal to or less than 10 moles of antibody-antigen binding sites of the antibody to 1 mole of human C5 in the body fluid.	Eculizumab is effective at a concentration yielding a ratio of 1.1 moles of antibody-antigen binding sites of the antibody to 1 mole of human C5.[2]
7. The antibody of claim 1 wherein the antibody is a humanized antibody.	Eculizumab is a humanized antibody (see Exhibits A and F).
9. The antibody of claim 1, wherein, when administered to a human patient via intravenous infusion, the antibody provides complete complement inhibition at dosages below 0.005 g/kg.	Eculizumab is administered intravenously (see Exhibit A) and inhibits complement activation at dosages below 0.005 g/kg.[3]
12. The antibody of claim 1 wherein the inhibition of complement activation in the human body fluid is measurable as a substantially complete blockade of C5a generation in the body fluid and a substantially complete blockade of complement hemolytic activity in the body fluid when the antibody is added to the body fluid at a concentration	Eculizumab is effective at a concentration yielding a ratio of 1.1 moles of antibody-antigen binding sites of the antibody to 1 mole of human C5 (see footnote 2 and Exhibit H).

[2] Complement C5 is present in plasma at a concentration of approximately 80 μg/mL (see Prodinger et al. in Fundamental Immunology, Fourth Edition, edited by William E. Paul, Chapter 29, page 969 (Lippincott-Raven Publishers, Philadelphia (1999)) (Exhibit I) which equates to a molar concentration of about 4.2×10^{-7} M (molecular weight of C5 is 190 kDa). An effective concentration of eculizumab in the serum of the PNH patients is ≥35 μg/mL (see Hill et al. (Exhibit F) and Hillmen et al. (Exhibit L) showing results of a clinical trial using Soliris™ (eculizumab) to treat PNH patients) which equates to a molar concentration of about 2.36×10^{-7} M antibody (molecular weight of eculizumab is 148 kDa), or 4.7×10^{-7} M antibody-antigen binding sites (two binding sites per molecule). This produces a 1.1:1 ratio of antibody-antigen binding sites to molecules of C5.

[3] Eculizumab levels of ≥35 μg/mL inhibit complement (see Hill et al. (Exhibit F)). For a typical person of 70 kg having a total blood volume of 5.6 L (see Vander et al., Human Physiology: The Mechanisms of Body Function, p. 246 (McGraw-Hill, 1970 (NY)) (Exhibit J)), the 35 μg/mL level of eculizumab equates to 0.0028 g/kg.

Applicable Claims of U.S. Patent No. 6,355,245	Manner in Which Each Claim Reads on Approved Product or Method of Using the Approved Product
yielding a ratio equal to or less than 3 moles of antibody-antigen binding sites of the antibody to 1 mole of human C5 in the body fluid.	
13. The antibody of claim 1, wherein, when administered to a human patient via intravenous infusion, the antibody provides therapeutically effective complement inhibition at dosages below 0.003 g/kg.	Eculizumab is administered intravenously (see Exhibit A) and inhibits complement activation at dosages below 0.003 g/kg (see footnote 3).
14. A sterile non-pyrogenic therapeutic agent comprising the antibody of claim 1 in a formulation suitable for administration to a human.	Soliris™ is a sterile non-pyrogenic formulation of eculizumab (see Exhibit A).
15. The therapeutic agent of claim 14 wherein the antibody is a humanized immunoglobulin.	Eculizumab is a humanized immunoglobulin (see Exhibit A).
17. The therapeutic agent of claim 14 wherein the antibody is made up of two or more heterodimeric subunits each containing one heavy and one light chain.	Eculizumab has two heterodimeric subunits each having a heavy chain and a light chain (see Exhibit A).
19. An isolated antigen binding protein comprising: 1) a variable light region CDR1 comprising an amino acid sequence corresponding to amino acid residues 26-36 of SEQ ID NO:8, 2) a variable light region CDR2 comprising an amino acid sequence corresponding to amino acid residues 52-58 of SEQ ID NO:8, 3) a variable light region CDR3 comprising an amino acid sequence corresponding to amino acid residues 91 through amino acid 99 of SEQ ID NO:8, 4) a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid residues 152 through amino acid 161 of SEQ ID NO:8, 5) a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid residues 176 through amino acid 192 of SEQ ID NO:8, 6) a variable heavy region CDR3 comprising an amino acid sequence corresponding to amino acid residues 225 through amino acid 237 of SEQ ID NO:8, said protein exhibiting specific binding to	The CDR sequences of eculizumab read on claim 19 (see Exhibit K). Eculizumab exhibits specific binding to human complement component C5, binds to the alpha chain of C5, inhibits complement activation in a human body fluid, and does not bind to free C5a as discussed above for claim 1.

Applicable Claims of U.S. Patent No. 6,355,245	Manner in Which Each Claim Reads on Approved Product or Method of Using the Approved Product
human complement component C5, said specific binding being targeted to the alpha chain of human complement component C5, wherein the protein inhibits complement activation in a human body fluid and does not specifically bind to the human complement activation product free C5a.	
23. An antibody comprising at least one antibody-antigen binding site, said antibody exhibiting specific binding to human complement component C5, said specific binding being targeted to the alpha chain of human complement component C5, wherein: (A) the antibody inhibits (i) C5b-9-mediated hemolysis and (ii) C5a generation in a fluid comprising human serum; and (B) the antibody does not specifically bind to the human complement activation product free C5a.	Eculizumab is an antibody with at least one antibody-antigen binding site, binds specifically to human complement C5, and more specifically to the alpha chain of C5 as discussed above for claim 1. A) Eculizumab inhibits C5b-9 mediated hemolysis (see Hillmen et al., N. Engl. J. Med. 355:1233-1243 (2006) (Exhibit G), especially Figure 1A, and Hillmen et al. N. Engl. J. Med. 350:552-559 (2004) (Exhibit L), especially Figure 2, which show results of a clinical trial of eculizumab (Soliris™) in PNH patients). B) 5G1.1 does not specifically bind to free C5a (see Example 13 of the '245 patent, especially column 51, lines 39-56).

10. Statement of Relevant Dates and Information Pursuant to 35 U.S.C. §156(g) (37 C.F.R. §1.740(a)(10))

BLA 125,166 was submitted and approved for Soliris™. The relevant dates are as follows:

(i)(A) The effective Date of the Investigational New Drug (IND) Application is June 27, 2003 and the IND Number is 11,075.[4]

(i)(B) The date on which the BLA was initially submitted is September 15, 2006 and the BLA Number is 125,166

(i)(C) The date on which the BLA was approved is March 16, 2007

[4] Earlier INDs were effective for eculizumab prior to June 27, 2003; however solely for convenience they are being ignored in this Application because the June 27, 2003 IND provides Applicant the maximum available patent term extension without needing to invoke the additional time period the earlier-filed INDs would provide.

11. Brief Description of Significant Activities Undertaken by Marketing Applicant During Applicable Regulatory Review Period and Respective Dates (37 C.F.R. §1.740(a)(11))

Attached as Exhibit M is a brief description of the significant activities undertaken by the marketing applicant with respect to Soliris™ during the regulatory review period for BLA 125,166.

12. Statement of Eligibility for Extension, Length of Extension Claimed and the Determination of Such Extension (37 C.F.R. §1.740(a)(12))

Applicant believes that U.S. Patent No. 6,355,245 is eligible for extension under 35 U.S.C. §156 because it satisfies all of the requirements for such extension as set forth below:

a. **35 U.S.C. §156(a), 37 C.F.R. §1.720**

U.S. Patent No. 6,355,245 claims a product.

b. **35 U.S.C. §156(a)(1)**

The term of U.S. Patent No. 6,355,245 will not have expired before submission of this application.

c. **35 U.S.C. §156(a)(2)**

The term of U.S. Patent No. 6,355,245 has never been extended under 35 U.S.C. §156(e)(1).

d. **35 U.S.C. §156(a)(3)**

This application for extension is submitted by an attorney for the owner of record in accordance with the requirements of 35 U.S.C. §156(d)(1)-(4) and rules of the U.S. Patent and Trademark Office.

e. **35 U.S.C. §156(a)(4)**

The Approved Product has been subject to a regulatory review period before its commercial marketing or use.

f. **35 U.S.C. §156(a)(5)(A)**

The commercial marketing or use of the Approved Product is the first permitted commercial marketing or use of the product under The Public Health and Welfare Act (42 U.S.C. §262), under which such regulatory review period occurred.

g. **35 U.S.C. §156(c)(4)**

No other patent has been extended for the same regulatory review period for the Approved Product.

In the opinion of the Applicant, U.S. 6,355,245 is entitled to an extension of 735 days, pursuant to 35 U.S.C. §156 and the implementing regulations, based upon the regulatory review period for the Approved Product.

The claimed length of this extension of 735 days was determined pursuant to 37 C.F.R. §1.775 as follows:

(1) The regulatory review period under 35 U.S.C. §156(g)(1)(B), which began on June 27, 2003, and ended on March 16, 2007, is the sum of computations in (i) and (ii) below:

(i) The period of review under 35 U.S.C. §156(g)(1)(B)(i) began on June 27, 2003, and ended on September 15, 2006, a period of 1176 days; and

(ii) The period of review under 35 U.S.C. §156(g)(1)(B)(ii) began on September 15, 2006, and ended on March 16, 2007, a period of 183 days;

the sum of (i) and (ii) is 1176 + 183 = 1359 days;

(2) The regulatory review period upon which the period of extension is calculated is the entire regulatory review period as determined in subparagraph 12(1) above (1359 days) less the sum of (i), (ii) and (iii) below:

(i) The number of days in the regulatory review period which were on or before the date on which the patent issued, March 12, 2002, which is zero (0) days, and

(ii) The number of days during which applicant did not act with due diligence, which is zero (0) days, and

(iii) One-half the number of days determined in subparagraph (12)(1)(i) (1176 /2 = 588 days;

1359 minus (i + ii + iii) is 1359 - (0 + 0 + 588) = 771 days;

(3) The number of days as determined in subparagraph 12(2) in its entirety (771), when added to the original term of the patent, would result in the date April 21, 2021;

(4) Fourteen (14) years when added to the date of approval (March 16, 2007) would result in the date March 16, 2021;

(5) The earlier date as determined in subparagraphs 12(3) and (12)(4) is March 16, 2021.

(6) Since the original patent issued after September 24, 1984, five (5) years

are added to the original expiration date of the patent, resulting in a date of March 12, 2024; and

(7) The earlier of the dates obtained in subparagraph 12(5) and in subparagraph 12(6) is March 16, 2021.

Therefore, the length of extension of patent term claimed by applicant is 735 days, which is the period of time needed to extend the original expiration of term of March 12, 2019, until March 16, 2021.

13. Statement of Acknowledgment of Duty to Disclose Material Information (37 C.F.R. §1.740(a)(13))

Applicant acknowledges a duty to disclose to the Director of the United States Patent and Trademark Office and the Secretary of Health and Human Services or the Secretary of Agriculture any information which is material to the determination of entitlement to the extension sought in this application.

14. Prescribed Fee (37 C.F.R. §1.740(a)(14))

Please charge Deposit Account No. 18-1945 in the amount of $1,120.00 as the fee covering the instant application for patent term extension as prescribed in 37 C.F.R. §1.20(j). The Commissioner is hereby authorized to charge any additional fees which may be required or credit any overpayment to Account No. 18-1945.

15. Contact Information (37 C.F.R. §1.740(a)(15))

All inquiries and correspondence relating to this application for patent term extension should be directed to:

Anita Varma, Esq.
Fish & Neave IP Group
Ropes & Gray LLP
One International Place
Boston, Massachusetts 02110-2624
Tel.: (617) 951-7000
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Stephen A. Saxe, Ph.D.
Associate General Counsel, Intellectual Property
Alexion Pharmaceuticals, Inc.
352 Knotter Drive
Cheshire, CT 06410
Tel.: (203) 271-8289
Fax: (203) 271-8195

16. Copies Enclosed (37 C.F.R. §1.740(b))

Three copies of the present application papers are enclosed.

Applicant is providing herewith in Exhibit N a power of attorney and general authority for the undersigned to execute this application on behalf of Alexion Pharmaceuticals, Inc.

Dated: 5/11/07

Respectfully submitted,

By Anita Varma

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Attorneys/Agents For Applicant

Attachments

Exhibit A: Soliris™ package insert as approved by the FDA

Exhibit B: Redacted FDA approval letter

Exhibit C: Copy of U.S. Patent No. 6,355,245

Exhibit D: Copy of maintenance fee receipt

Exhibit E: Copy of Certificate of Correction for U.S. Patent No. 6,355,245

Exhibit F: Hill et al., Blood 106:2559-2565 (2005)

Exhibit G: Hillmen et al., N. Engl. J. Med. 355:1233-1243 (2006)

Exhibit H: Thomas et al., Molecular Immunology 33:1389-1401 (1996)

Exhibit I: Prodinger et al., Chapter 29 (pp. 967-995) in: Fundamental Immunology, Fourth Edition, edited by William E. Paul, Lippincott-Raven Publishers, Philadelphia (1999)

Exhibit J: Vander et al., p. 246, in Human Physiology: The Mechanisms of Body Function, McGraw-Hill Book Company (1970)

Exhibit K: Sequences of heavy and light chains of eculizumab

Exhibit L: Hillmen et al., N. Engl. J. Med. 350:552-559 (2004)

Exhibit M: Description of significant activities undertaken during the regulatory review period for Soliris™ and applicable dates for such activities

Exhibit N: Power of Attorney and General Authority from Assignee

HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use Soliris safely and effectively. See full prescribing information for Soliris.

Soliris™ (eculizumab),

Concentrated solution for intravenous infusion
Initial U.S. Approval: 2007

WARNING: SERIOUS MENINGOCOCCAL INFECTIONS

See full prescribing information for complete boxed warning

Soliris increases the risk of meningococcal infections (5.1)

- **Vaccinate patients with a meningococcal vaccine at least 2 weeks prior to receiving the first dose of Soliris; revaccinate according to current medical guidelines for vaccine use**
- **Monitor patients for early signs of meningococcal infections, evaluate immediately if infection is suspected, and treat with antibiotics if necessary.**

INDICATIONS AND USAGE

Soliris is a complement inhibitor indicated for the treatment of patients with paroxysmal nocturnal hemoglobinuria (PNH) to reduce hemolysis (1).

DOSAGE AND ADMINISTRATION

Dosage Regimen: (2.1)

- 600 mg via 35 minute intravenous infusion every 7 days for the first 4 weeks, followed by
- 900 mg for the fifth dose 7 days later, then
- 900 mg every 14 days thereafter

Administration: (2.2, 2.3)

- Do not administer as an intravenous push or bolus.
- Dilute to a final concentration of 5 mg/mL prior to administration.
- Administer by intravenous infusion over 35 minutes.

DOSAGE FORMS AND STRENGTHS

300 mg single-use vials each containing 30 mL of 10 mg/mL sterile, preservative-free solution (3).

CONTRAINDICATIONS

Do not initiate Soliris therapy in patients:

- with unresolved serious *Neisseria meningitidis* infection (4).
- who are not currently vaccinated against *Neisseria meningitidis* (4).

WARNINGS AND PRECAUTIONS

- Other Infections: Use caution when administering Soliris to patients with any systemic infection (5.2).
- Monitoring After Soliris Discontinuation: Soliris increases the number of PNH red blood cells (RBCs). All patients who discontinue Soliris therapy should be monitored for signs and symptoms of intravascular hemolysis, including evaluation of serum lactate dehydrogenase (LDH) levels (5.3).

ADVERSE REACTIONS

The most frequently reported adverse reactions (≥10% overall and greater than placebo) are: headache, nasopharyngitis, back pain and nausea (6).

To report SUSPECTED ADVERSE REACTIONS, contact Alexion Pharmaceuticals, Inc. at 1-888-SOLIRIS (1-888-765-4747) or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch.

See 17 PATIENT COUNSELING INFORMATION AND MEDICATION GUIDE

Revised: 3/2007

FULL PRESCRIBING INFORMATION: CONTENTS*

*Sections or subsections omitted from the full prescribing information are not listed.

Exhibit A

FULL PRESCRIBING INFORMATION

WARNING: SERIOUS MENINGOCOCCAL INFECTION

Soliris increases the risk of meningococcal infections (5.1)

- **Vaccinate patients with a meningococcal vaccine at least 2 weeks prior to receiving the first dose of Soliris; revaccinate according to current medical guidelines for vaccine use**
- **Monitor patients for early signs of meningococcal infections, evaluate immediately if infection is suspected, and treat with antibiotics if necessary.**

1 INDICATIONS AND USAGE

Soliris is indicated for the treatment of patients with paroxysmal nocturnal hemoglobinuria (PNH) to reduce hemolysis.

2 DOSAGE AND ADMINISTRATION

Patients must be administered a meningococcal vaccine at least two weeks prior to initiation of Soliris therapy and revaccinated according to current medical guidelines for vaccine use. [*see Warnings and Precautions (5.1)*].

2.1 Recommended Dosage Regimen

Soliris therapy consists of:

- 600 mg every 7 days for the first 4 weeks, followed by
- 900 mg for the fifth dose 7 days later, then
- 900 mg every 14 days thereafter.

Soliris should be administered at the recommended dosage regimen time points, or within two days of these time points. [*see Warnings and Precautions (5.5)*]

2.2 Preparation for Administration

Soliris must be diluted to a final admixture concentration of 5 mg/mL using the following steps:

- Withdraw the required amount of Soliris from the vial into a sterile syringe.
- Transfer the recommended dose to an infusion bag.
- Dilute Soliris to a final concentration of 5 mg/mL by adding the appropriate amount (equal volume of diluent to drug volume) of 0.9% Sodium Chloride Injection, USP; 0.45% Sodium Chloride Injection, USP; 5% Dextrose in Water Injection, USP; or Ringer's Injection, USP to the infusion bag.

The final admixed Soliris 5 mg/mL infusion volume is 120 mL for 600 mg doses or 180 mL for 900 mg doses. Gently invert the infusion bag containing the diluted Soliris solution to ensure thorough mixing of the product and diluent. Discard any unused portion left in a vial, as the product contains no preservatives.

Prior to administration, the admixture should be allowed to adjust to room temperature [18°-25° C, 64-77° F]. The admixture must not be heated in a microwave or with any heat source other than ambient air temperature. The Soliris admixture should be inspected visually for particulate matter and discoloration prior to administration.

2.3 Administration

Do Not Administer As An Intravenous Push Or Bolus Injection

The Soliris admixture should be administered by intravenous infusion over 35 minutes via gravity feed, a syringe-type pump, or an infusion pump. Admixed solutions of Soliris are stable for 24 hours at 2-8° C (36-46° F) and at room temperature.

If an adverse reaction occurs during the administration of Soliris, the infusion may be slowed or stopped at the discretion of the physician. If the infusion is slowed, the total infusion time should not exceed two hours. Monitor the patient for at least one hour following completion of the infusion for signs or symptoms of an infusion reaction.

3 DOSAGE FORMS AND STRENGTHS

Soliris is supplied as 300 mg single-use vials each containing 30 mL of 10 mg/mL sterile, preservative-free eculizumab solution.

4 CONTRAINDICATIONS

Do not initiate Soliris therapy in patients:

- with unresolved serious *Neisseria meningitidis* infection.
- who are not currently vaccinated against *Neisseria meningitidis*.

5 WARNINGS AND PRECAUTIONS

5.1 Serious Meningococcal Infections

The use of Soliris increases a patient's susceptibility to serious meningococcal infections (septicemia and/or meningitis). All patients without a history of prior meningococcal vaccination must receive the meningococcal vaccine at least 2 weeks prior to receiving the first dose of Soliris and revaccinated according to current medical guidelines for vaccine use. Quadravalent, conjugated meningococcal vaccines are strongly recommended. Vaccination may not prevent meningococcal infections.

All patients must be monitored for early signs and symptoms of meningococcal infections and evaluated immediately if an infection is suspected. Physicians should strongly consider discontinuation of Soliris during the treatment of serious meningococcal infections.

In clinical studies, 2 out of 196 PNH patients developed serious meningococcal infections while receiving treatment with Soliris; both had been vaccinated. [*see Adverse Reactions (6.1)*].

5.2 Other Infections

Soliris blocks terminal complement; therefore patients may have increased susceptibility to infections, especially with encapsulated bacteria. Use caution when administering Soliris to patients with any systemic infection.

5.3 Monitoring After Soliris Discontinuation

Since Soliris therapy increases the number of PNH cells [in study 1, the proportion of PNH RBCs increased among Soliris-treated patients by a median of 28% from baseline (range from -25% to 69%)], patients who discontinue treatment with Soliris may be at increased risk for serious hemolysis. Serious hemolysis is identified by serum LDH levels greater than the pre-treatment level, along with any of the following: greater than 25% absolute decrease in PNH clone size (in the absence of dilution due to transfusion) in one week or less; a hemoglobin level of <5 gm/dL or a decrease of >4 gm/dL in one week or less; angina; change in mental status; a 50% increase in serum creatinine level; or thrombosis. Monitor any patient who discontinues Soliris for at least 8 weeks to detect serious hemolysis and other reactions.

If serious hemolysis occurs after Soliris discontinuation, consider the following procedures/treatments: blood transfusion (packed RBCs), or exchange transfusion if the PNH RBCs are >50% of the total RBCs by flow cytometry; anticoagulation; corticosteroids; or reinstitution of Soliris.

In clinical studies, 16 of 196 PNH patients discontinued treatment with Soliris. Patients were followed for evidence of worsening hemolysis and no serious hemolysis was observed.

5.4 Thrombosis Prevention and Management

The effect of withdrawal of anticoagulant therapy during Soliris treatment has not been established. Therefore, treatment with Soliris should not alter anticoagulant management.

5.5 Laboratory Monitoring

Serum LDH levels increase during hemolysis and may assist in monitoring Soliris effects, including the response to discontinuation of therapy. In clinical studies, six patients achieved a reduction in serum LDH levels only after a decrease in the Soliris dosing interval from 14 to 12 days. All other patients achieved a reduction in serum LDH levels with the 14 day dosing interval [*see Clinical Pharmacology (12.2) and Clinical Studies (14)*].

5.6 Infusion Reactions

As with all protein products, administration of Soliris may result in infusion reactions, including anaphylaxis or other hypersensitivity reactions. In clinical trials, no PNH patients experienced an infusion reaction which required discontinuation of Soliris. Soliris administration should be interrupted in all patients experiencing severe infusion reactions and appropriate medical therapy administered.

6 ADVERSE REACTIONS

6.1 Clinical Trial Experience

Meningococcal infections are the most important adverse reactions experienced by patients receiving Soliris therapy. In PNH clinical studies, two patients experienced meningococcal sepsis. Both patients had previously received a meningococcal vaccine. In clinical studies among patients without PNH, meningococcal meningitis occurred in an unvaccinated patient [*see Warnings and Precautions (5.1)*].

The data described below reflect exposure to Soliris in 196 adult patients with PNH, age 18-85, of whom 55% were female. All had signs or symptoms of intravascular hemolysis. Soliris was studied in a placebo-controlled clinical study (in which 43 patients received Soliris and 44, placebo); a single arm clinical study and a long term extension study. 182 patients were exposed for greater than one year. All patients received the recommended Soliris dose regimen.

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not

reflect the rates observed in practice. Table 1 summarizes the adverse reactions that occurred at a numerically higher rate in the Soliris group than the placebo group and at a rate of 5% or more among patients treated with Soliris.

TABLE 1

ADVERSE REACTIONS REPORTED IN 5% OR MORE OF SOLIRIS TREATED PATIENTS AND GREATER THAN PLACEBO IN THE CONTROLLED CLINICAL STUDY

Reaction	Soliris N = 43 N (%)	Placebo N = 44 N (%)
Headache	19 (44)	12 (27)
Nasopharyngitis	10 (23)	8 (18)
Back pain	8 (19)	4 (9)
Nausea	7 (16)	5 (11)
Fatigue	5 (12)	1 (2)
Cough	5 (12)	4 (9)
Herpes simplex infections	3 (7)	0
Sinusitis	3 (7)	0
Respiratory tract infection	3 (7)	1 (2)
Constipation	3 (7)	2 (5)
Myalgia	3 (7)	1 (2)
Pain in extremity	3 (7)	1 (2)
Influenza-like illness	2 (5)	1 (2)

In the placebo-controlled clinical study, serious adverse reactions occurred among 4 (9%) patients receiving Soliris and 9 (21%) patients receiving placebo. The serious reactions included infections and progression of PNH. No deaths occurred in the study and no patients receiving Soliris experienced a thrombotic event; one thrombotic event occurred in a patient receiving placebo.

Among 193 patients with PNH treated with Soliris in the single arm, clinical study or the follow-up study, the adverse reactions were similar to those reported in the placebo-controlled clinical study. Serious adverse reactions occurred among 16% of the patients in these studies. The most common serious adverse reactions were: viral infection (2%), headache (2%), anemia (2%), and pyrexia (2%).

6.2 Immunogenicity

As with all proteins there is a potential for immunogenicity. Low titers of antibodies to Soliris were detected in 3/196 (2%) of all PNH patients treated with Soliris. No apparent correlation of antibody development to clinical response was observed. The immunogenicity data reflect the percentage of patients whose test results were considered positive for antibodies to Soliris in an enzyme linked immunosorbent assay (ELISA) and are highly dependent on the sensitivity and specificity of the assay. Additionally, the observed incidence of antibody positivity in the assay may be influenced by several factors including sample handling, timing of sample collection, concomitant medications and underlying disease. For these reasons, comparison of the incidence of antibodies to Soliris with the incidence of antibodies to other products may be misleading.

7 DRUG INTERACTIONS

Drug interaction studies have not been performed with Soliris.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Pregnancy Category C:

PNH is a serious illness. Pregnant women with PNH and their fetuses have high rates of morbidity and mortality during pregnancy and the postpartum period. There are no adequate and well-controlled studies of Soliris in pregnant women. Soliris, a recombinant IgG molecule (humanized anti-C5 antibody), is expected to cross the placenta. Animal studies using a mouse analogue of the Soliris molecule (murine anti-C5 antibody) showed increased rates of developmental abnormalities and an increased rate of dead and moribund offspring at doses 2-8 times the human dose. Soliris should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Animal reproduction studies were conducted in mice using doses of a murine anti-C5 antibody that approximated 2-4 times (low dose) and 4-8 times (high dose) the recommended human Soliris dose, based on a body weight comparison. When animal exposure to the antibody occurred in the time period from before mating until early gestation, no decrease in fertility or reproductive performance was observed. When maternal exposure to the antibody occurred during organogenesis, two cases of retinal dysplasia and one case of umbilical hernia were observed among 230 offspring born to mothers exposed to the higher antibody dose; however, the exposure did not increase fetal loss or neonatal death. When maternal exposure to the antibody occurred in the time period from implantation through weaning, a higher number of male offspring became moribund or died (1/25 controls, 2/25 low dose group, 5/25 high dose group). Surviving offspring had normal development and reproductive performance.

8.2 Labor and Delivery

No information is available on the effects of Soliris during labor and delivery.

8.3 Nursing Mothers

It is not known whether Soliris is secreted into human milk. IgG is excreted in human milk, so it is expected that Soliris will be present in human milk. However, published data suggest that breast milk antibodies do not enter the neonatal and infant circulation in substantial amounts. Caution should be exercised when Soliris is administered to a nursing woman. The unknown risks to the infant from gastrointestinal or limited systemic exposure to Soliris should be weighed against the known benefits of breastfeeding.

8.4 Pediatric Use

The safety and effectiveness of Soliris therapy in pediatric patients below the age of 18 have not been established.

8.5 Geriatric Use

In PNH studies, 15 patients 65 years of age or older were treated with Soliris. Although there were no apparent age-related differences observed in these studies, the number of patients aged 65 and over is not sufficient to determine whether they respond differently from younger patients.

10 OVERDOSAGE

No cases of Soliris overdose have been reported during clinical studies.

11 DESCRIPTION

Soliris is a formulation of eculizumab which is a recombinant humanized monoclonal $IgG_{2/4}\kappa$ antibody produced by murine myeloma cell culture and purified by standard bioprocess technology. Eculizumab contains human constant regions from human IgG2 sequences and human IgG4 sequences and murine complementarity-determining regions grafted onto the human framework light- and heavy-chain variable regions. Eculizumab is composed of two 448 amino acid heavy chains and two 214 amino acid light chains and has a molecular weight of approximately 148 kDa.

Soliris is a sterile, clear, colorless, preservative-free 10 mg/mL solution for intravenous infusion and is supplied in 30-mL single-use vials. The product is formulated at pH 7 and each vial contains 300 mg of eculizumab, 13.8 mg sodium phosphate monobasic, 53.4 mg sodium phosphate dibasic, 263.1 mg sodium chloride, 6.6 mg polysorbate 80 (vegetable origin) and Water for Injection, USP.

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

Eculizumab, the active ingredient in Soliris, is a monoclonal antibody that specifically binds to the complement protein C5 with high affinity, thereby inhibiting its cleavage to C5a and C5b and preventing the generation of the terminal complement complex C5b-9. Soliris inhibits terminal complement mediated intravascular hemolysis in PNH patients.

A genetic mutation in PNH patients leads to the generation of populations of abnormal RBCs (known as PNH cells) that are deficient in terminal complement inhibitors, rendering PNH RBCs sensitive to persistent terminal complement-mediated destruction. The destruction and loss of these PNH cells (intravascular hemolysis) results in low RBC counts (anemia), and also fatigue, difficulty in functioning, pain, dark urine, shortness of breath, and blood clots.

12.2 Pharmacodynamics

In the placebo-controlled clinical study, Soliris when administered as recommended reduced hemolysis as shown by the reduction of serum LDH levels from 2200 ± 1034 U/L (mean ± SD) at baseline to 700 ± 388 U/L by week one and maintained the effect through the end of the study at week 26 (327 ± 433 U/L). In the single arm clinical study, Soliris maintained this effect through 52 weeks [*see Clinical Studies (14)*].

12.3 Pharmacokinetics

A population PK analysis with a standard 1-compartmental model was conducted on the multiple dose PK data from 40 PNH patients receiving the recommended Soliris regimen [*see Dosage and Administration (2.1)*]. In this model, the clearance of Soliris for a typical PNH patient weighing 70 kg was 22 mL/hr and the volume of distribution was 7.7 L. The half-life was 272 ± 82 hrs (mean ± SD). The mean observed peak and trough serum concentrations of Soliris by week 26 were 194 ± 76 mcg/mL and 97 ± 60 mcg/mL, respectively.

Studies have not been conducted to evaluate the PK of Soliris in special patient populations identified by gender, race, age (pediatric or geriatric), or the presence of renal or hepatic impairment.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

Long-term animal studies have not been conducted to evaluate the carcinogenic and genotoxic potential of Soliris. Effects of Soliris upon fertility have not been studied in animals. Intravenous injections of male and female mice with a murine anti-C5 antibody at up to 4-8 times the equivalent of the clinical dose of Soliris had no adverse effects on mating or fertility.

14 CLINICAL STUDIES

The safety and efficacy of Soliris in PNH patients with hemolysis were assessed in a randomized, double-blind, placebo-controlled 26 week study (Study 1); PNH patients were also treated with Soliris in a single arm 52 week study (Study 2); and in a long term extension study. Patients received meningococcal vaccination prior to receipt of Soliris. In all studies, the dose of Soliris was 600 mg study drug every 7 ± 2 days for 4 weeks, followed by 900 mg 7 ± 2 days later, then 900 mg every 14 ± 2 days for the study duration. Soliris was administered as an intravenous infusion over 25 - 45 minutes.

Study 1:

PNH patients with at least four transfusions in the prior 12 months, flow cytometric confirmation of at least 10% PNH cells and platelet counts of at least 100,000/microliter were randomized to either Soliris (n = 43) or placebo (n = 44). Prior to randomization, all patients underwent an initial observation period to confirm the need for RBC transfusion and to identify the hemoglobin concentration (the "set-point") which would define each patient's hemoglobin stabilization and transfusion outcomes. The hemoglobin set-point was less than or equal to 9 g/dL in patients with symptoms and was less than or equal to 7 g/dL in patients without symptoms. Endpoints related to hemolysis included the numbers of patients achieving hemoglobin stabilization, the number of RBC units transfused, fatigue, and health-related quality of life. To achieve a designation of hemoglobin stabilization, a patient had to maintain a hemoglobin concentration above the hemoglobin set-point and avoid any RBC transfusion for the entire 26 week period. Hemolysis was monitored mainly by the measurement of serum LDH levels, and the proportion of PNH RBCs was monitored by flow cytometry. Patients receiving anticoagulants and systemic corticosteroids at baseline continued these medications.

Major baseline characteristics were balanced (see table 2).

TABLE 2
STUDY 1 PATIENT BASELINE CHARACTERISTICS

Parameter	Study 1 Placebo N = 44	Study 1 Soliris N = 43
Mean age (SD)	38 (13)	42 (16)
Gender - female (%)	29 (66)	23 (54)
History of aplastic anemia or myelodysplastic syndrome (%)	12 (27)	8 (19)
Patients with history of thrombosis (events)	8 (11)	9 (16)
Concomitant anticoagulants (%)	20 (46)	24 (56)
Concomitant steroids/immunosuppressant treatments (%)	16 (36)	14 (33)
Packed RBC units transfused per patient in previous 12 months (median (Q1,Q3))	17 (14, 25)	18 (12, 24)
Mean hgb level (g/dL) at setpoint (SD)	8 (1)	8 (1)
Pre-treatment LDH levels (median, U/L)	2,234	2,032
Free hemoglobin at baseline (median, mg/dL)	46	41

Patients treated with Soliris had significantly reduced ($p < 0.001$) hemolysis resulting in improvements in anemia as indicated by increased hemoglobin stabilization and reduced need for RBC transfusions compared to placebo treated patients (see table 3). These effects were seen among patients within each of the three pre-study RBC transfusion strata (4 - 14 units; 15 - 25 units; > 25 units). After 3 weeks of Soliris treatment, patients reported less fatigue and improved health-related quality of life. Because of the study sample size and duration, the effects of Soliris on thrombotic events could not be determined.

TABLE 3
STUDY 1 RESULTS

	Placebo N = 44	Soliris N = 43
Percentage of patients with stabilized hemoglobin levels	0	49
Median Packed RBC units transfused per patient (range)	10 (2 – 21)	0 (0 – 16)
Transfusion avoidance (%)	0	51
LDH levels at end of study (median, U/L)	2,167	239
Free hemoglobin at end of study (median, mg/dL)	62	5

Study 2 and Extension Study:
PNH patients with at least one transfusion in the prior 24 months and at least 30,000 platelets/microliter received Soliris over a 52-week period. Concomitant medications included anti-thrombotic agents in 63% of the patients and systemic corticosteroids in 40% of the patients. Overall, 96 of the 97 enrolled patients completed the study (one patient died following a thrombotic event). A reduction in intravascular hemolysis as measured by serum LDH levels was sustained for the treatment period and resulted in a reduced need for RBC transfusion and less fatigue. 187 Soliris-treated PNH patients were enrolled in a long term extension study. All patients sustained a reduction in intravascular hemolysis over a total Soliris exposure time ranging from 10 to 54 months. There were fewer thrombotic events with Soliris treatment than during the same period of time prior to treatment. However, the majority of patients received concomitant anticoagulants; the effects of anticoagulant withdrawal during Soliris therapy was not studied [*see Warnings and Precautions (5.4)*].

16 HOW SUPPLIED / STORAGE AND HANDLING

Soliris (eculizumab) is supplied as 300 mg single-use vials containing 30 mL of 10 mg/mL sterile, preservative-free Soliris solution per vial.

Soliris vials must be stored in the original carton until time of use under refrigerated conditions at 2-8° C (36-46° F) and protected from light. Do not use beyond the expiration date stamped on the carton. Refer to *Dosage and Administration* (2) for information on the stability and storage of diluted solutions of Soliris.

DO NOT FREEZE. DO NOT SHAKE.

NDC 25682-001-01 Single unit 300 mg carton: Contains one (1) 30 mL vial of Soliris (10 mg/mL).

17 PATIENT COUNSELING INFORMATION

See Medication Guide.

Prior to treatment, patients should fully understand the risks and benefits of Soliris, in particular the risk of meningococcal infection. Ensure that patients receive the Medication Guide.

Patients should be informed that they are required to receive a meningococcal vaccination at least 2 weeks prior to receiving the first dose of Soliris, if they have not previously been vaccinated. They are required to be revaccinated according to current medical guidelines for meningococcal vaccine use while on Soliris therapy. Patients should also be informed that vaccination may not prevent meningococcal infection. Patients should be educated about any of the signs and symptoms of meningococcal infection, and strongly advised to seek immediate medical attention if these signs or symptoms occur. These signs and symptoms are as follows:

- moderate to severe headache with nausea or vomiting
- moderate to severe headache and a fever
- moderate to severe headache with a stiff neck or stiff back
- fever of 103° F (39.4° C) or higher
- fever and a rash
- confusion
- severe muscle aches with flu-like symptoms, and eyes sensitive to light

Patients should be informed that they would be provided with the Patient Safety Card that they should carry with them at all times. This card describes symptoms which, if experienced, should prompt the patient to immediately seek medical evaluation.

Patients should be informed that there is a potential for serious hemolysis when Soliris is discontinued and that they will be monitored by their healthcare professional for at least 8 weeks following Soliris discontinuation.

Manufactured by:

Alexion Pharmaceuticals, Inc.

352 Knotter Drive

Cheshire, CT 06410 USA

US License Number 1743

MEDICATION GUIDE

Soliris (eculizumab)
(so-leer-is)

Read the Medication Guide before you start Soliris and before each dose (infusion). This Medication Guide does not take the place of talking with your doctor about your condition or your treatment. Talk to your doctor if you have any questions about your treatment with Soliris.

What Is The Most Important Information I Should Know About Soliris?

Soliris is a medicine that affects your immune system. Soliris can lower the ability of your immune system to fight infections.

- **Soliris increases your chance of getting serious and life-threatening meningococcal infections.**

1. **You must receive a meningococcal vaccine at least 2 weeks before your first dose of Soliris unless you have already had this vaccine.**

2. **If you had a meningococcal vaccine in the past, you might need a booster dose before starting Soliris.** Your doctor will decide if you need another dose of a meningococcal vaccine.

3. **A meningococcal vaccine does not prevent all meningococcal infections. You must be aware of the following signs and symptoms of a meningococcal infection:**

    - **moderate to severe headache with nausea or vomiting**
    - **moderate to severe headache and a fever**
    - **moderate to severe headache with a stiff neck or stiff back**
    - **fever of 103° F (39.4° C) or higher**
    - **fever and a rash**
    - **confusion**
    - **severe muscle aches with flu-like symptoms, and eyes sensitive to light**

Call your doctor or get emergency medical care right away if you have any of these symptoms.

You will receive a Patient Safety Card that lists these symptoms and what to do if you have them. Carry it with you at all times. You will need to show the card to any healthcare provider that treats you.

What Is Soliris?

Soliris is a medicine called a monoclonal antibody. Soliris is used for the treatment of patients with a disease that affects red blood cells called Paroxysmal Nocturnal Hemoglobinuria (PNH).

Soliris works by blocking part of your immune system. This can help your PNH symptoms but it can also increase your chance for infection. **It is important that you:**

- have all recommended immunizations and vaccines before you start Soliris
- stay up-to-date with all recommended immunizations and vaccines during treatment with Soliris

Who Should Not Receive Soliris?

Do not receive Soliris if you:

- have a meningococcal infection
- have not been vaccinated with, or you are not up-to-date with a meningococcal vaccine. See "What is the most important information about Soliris?"

Tell your doctor if you:

- have an infection or fever
- are pregnant, become pregnant, or are breastfeeding. Soliris has not been studied in pregnant or nursing women.

How Do I Receive Soliris?

- Soliris is given through a vein (I.V. infusion) over 35 minutes.
- You will usually receive a Soliris infusion:
  - every 7 days for five weeks, then
  - every 14 days
- Following each infusion, you may be monitored for one hour for allergic reactions.

What If I Miss a Dose or Stop Soliris Treatment?

- If you forget or miss a Soliris infusion, call your doctor right away.
- Stopping treatment with Soliris may cause a sudden and serious breakdown of your red blood cells. Symptoms or problems from red blood cell breakdown include:
  - a large drop in your red blood cell count causing anemia
  - confusion
  - chest pain
  - kidney problems
  - blood clots
- Your doctor will need to monitor you closely for at least 8 weeks after stopping Soliris.

What Are The Possible Side Effects With Soliris?

Serious side effects with Soliris include:

- **serious and life-threatening infections.** See "What is the most imortant information I should know about Soliris?"

Common side effects with Soliris include:

- headaches
- runny nose and colds
- sore throat
- back pain
- nausea

Call your doctor if you have any of these side effects. These are not all the side effects with Soliris. Ask your doctor for more information.

General Information About Soliris

Medicines are sometimes prescribed for conditions other than those listed in a Medication Guide. If you have any concerns about Soliris, ask your doctor. Your doctor or pharmacist can give you information about Soliris that was written for health care professionals.

Soliris contains eculizumab in a solution of water, polysorbate, sodium phosphate and sodium chloride.

Manufactured by Alexion Pharmaceuticals, Inc., 352 Knotter Drive, Cheshire, CT 06410 USA.

Revised: March 2007

This Medication Guide has been approved by the U.S. Food and Drug Administration

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
Food and Drug Administration
Rockville, MD 20857

MAR 16 2007

Our STN: BL 125166/0

Alexion Pharmaceuticals
Attention: Nancy Motola, Ph.D., RAC
Senior Vice President, Regulatory and Quality
352 Knotter Drive
Cheshire, CT 06410

Dear Dr. Motola:

We are issuing Department of Health and Human Services U.S. License No. 1743 to Alexion Pharmaceuticals, Inc., Cheshire, Connecticut, under the provisions of section 351(a) of the Public Health Service Act controlling the manufacture and sale of biological products. The license authorizes you to introduce or deliver for introduction into interstate commerce, those products for which your company has demonstrated compliance with establishment and product standards.

Under this license, you are approved to manufacture the product eculizumab, which is indicated for the treatment of paroxysmal nocturnal hemoglobinuria to reduce hemolysis.

You are approved to manufacture eculizumab drug substance at Lonza Biologics, PLC in Portsmouth, NH. The final formulated product will be manufactured, filled, labeled, and packaged at [redacted]. You may label your product with the proprietary name Soliris™ and will market it in 300 mg vials.

The final printed labeling (FPL) must be identical to the enclosed labeling. Marketing product with FPL that is not identical to the approved labeling text may render the product misbranded and an unapproved new drug.

The dating period for eculizumab drug product shall be 24 months from the date of manufacture when stored at 2-8°C. The date of manufacture shall be defined as the date of final sterile filtration of the formulated drug product. The dating period for your drug substance shall be 18 months when stored at 2-8°C.

You currently are not required to submit samples of future lots of eculizumab to the Center for Drug Evaluation and Research (CDER) for release by the Director, CDER, under 21 CFR 610.2. We will continue to monitor compliance with 21 CFR 610.1 requiring completion of tests for conformity with standards applicable to each product prior to release of each lot.

Exhibit B

You must submit information to your biologics license application for our review and written approval under 21 CFR 601 12 for any changes in the manufacturing, testing, packaging or labeling of eculizumab, or in the manufacturing facilities.

We acknowledge your written commitments as described in your letter of February 22, 2007, March 12, 2007 and March 12, 2007 as outlined below:

Postmarketing Studies subject to reporting requirements of 21 CFR 601.70.

1. To evaluate long-term safety of eculizumab by analyzing outcomes in the Soliris Safety Registry for a time period of no less than five years. At the end of the five year period, a study report will be submitted to the Biological License Application (BLA) that describes the major safety findings from the registry program, including the specific items listed below and proposing labeling changes as appropriate. Additionally, annual interim reports will be submitted to the BLA, along with expedited reports as specified below. The protocol for addressing this PMC will be submitted to the IND by May 31, 2007, and the five year study report will be submitted by June 30, 2012. All patients within the registry will be followed for the occurrence of:

    a. Serious infections, defined as infections necessitating or prolonging hospitalization or resulting in death. Alexion commits to collecting follow-up information from these patients to assess the nature of the serious infection, the duration of hospitalization, the major features of the clinical course and the survival status. Expedited reporting (15 day telephone or facsimile Medwatch communication) will be provided for the occurrence of these serious infections.

    b. Malignancy, including the nature of the malignancy and the survival status of patients who develop a malignancy;

    c. Use of eculizumab among pediatric patients under 16 years of age, to include collection of eculizumab dosage information, as well as the same information being required for adult patients in the registry;

    d. Pregnancy, including the clinical course of each pregnancy and the detection of congenital abnormalities among babies born to the women exposed to eculizumab during the pregnancy.

    e. Thrombotic events, including the nature of the event, the clinical outcome as well as the anticoagulant management prior to and after the event.

2. To submit a comprehensive description of the Soliris Guardian Program Risk Minimization Action Plan (RiskMAP), including all items listed below. Fulfillment of this post-marketing commitment will be contingent upon FDA concurrence upon the expectations of the Soliris Guardian Program. Submission of all items listed below will occur no later than May 18, 2007. The submitted information will include:

   a. A final version of the Soliris Guardian Program document that, in addition to any other items, provides information fully consistent with the approved prescribing information.

   b. A copy of all educational materials to be provided as part of the program, including but not limited to all components of the Soliris Starter Kit, the Drug Fact Sheet and patient-health care provider documents relating to the Soliris Safety Registry.

   c. A commitment to develop and submit a protocol (with the understanding that this protocol may need modification based upon FDA review findings) that uses surveys of health care providers and patients to assess compliance with the vaccination requirements as well as their knowledge of the risks of eculizumab and the need for vaccination.

   d. A copy of the Soliris Safety Registry protocol and any supportive documents to be provided to health care providers and patients. These documents may need modification based upon FDA review findings. The submitted protocol will indicate that the Registry will continue until Alexion receives written concurrence from FDA to terminate the registry. The protocol will include collection of the occurrence of the following events:

      i. Death;

      ii. Meningococcal vaccination (type and dates of all vaccinations);

      iii. eculizumab administration dates, at designated time points to establish initiation and termination of therapy as well as to correlate eculizumab administration with the fatalities and the events listed below:

         - All meningococcal infections causing sepsis or meningitis
         - Other serious infections
         - Malignancy, including the nature of the malignancy and the survival status of patients who develop a malignancy;
         - Use of eculizumab among pediatric patients under 16 years of age, to include collection of eculizumab dosage information, as well as the required information for adult patients in the registry;
         - Use of eculizumab by indication;

- Pregnancy, including the clinical course of each pregnancy and the detection of congenital abnormalities among off-spring of the women exposed to eculizumab during a pregnancy and;
- Serious hemolysis, as defined by specific criteria.

e. A commitment to submit quarterly RiskMAP reports for the first year and annual reports thereafter (unless FDA provides written request for more frequent submissions) summarizing all information relating to the Soliris Guardian Program. The reports should include the following:

i. An analysis of all cases of the following, including a root cause analysis and factors that might have contributed to serious outcomes:
- Meningococcal sepsis or meningitis, including the timing of all vaccinations relative to administration of Soliris
- Other infections (with serious outcomes)
- All deaths
- Cases of serious hemolysis and all cases of hemolysis with serious outcomes

ii. Soliris use patterns including indication for use;

iii. Extent of compliance with RiskMAP requirements such as the percentage of patients that were vaccinated prior to receiving eculizumab and the percentage of patients that were re-vaccinated at 3-year or 10-year intervals (as applicable);

iv. Analysis of all cumulative data collected in the Soliris Safety Registry;

v. Results from all health care provider and patient surveys, including:

- Any known data about patients or physicians who refused to participate in the surveys
- Any known data about survey participants who are considered "lost" (drop-outs).

3. To conduct a randomized, controlled clinical study to assess the effects of anticoagulant withdrawal among PNH patients receiving eculizumab. This study will randomize at least 100 anticoagulated patients to either continue or discontinue anticoagulation therapy. The major outcomes will assess the safety of discontinuation of anticoagulant therapy while continuing eculizumab, especially with respect to providing important evidence regarding major bleeding and that this discontinuation does not increase the risk for occurrence of thrombotic events in these patients. A full study report and data from this study will be submitted to the BLA and may include a label revision, contingent upon the importance of the study results. The study protocol will be submitted to the investigational new drug application (IND) by June 30, 2007 and patient accrual completed no later than June 1, 2009. A final study report will be submitted no later than March 31, 2014.

4. To develop a validated and quantitative assay for the measurement of human anti-human antibodies (HAHA) for the detection of antibody formation to eculizumab. This assay will assess potential immune responses to the whole eculizumab molecule. Description of the validated assay will be submitted to the BLA as a CBE 30 by July 9, 2008.

5. To develop a validated and sensitive assay for the measurement of neutralizing HAHA to eculizumab. Alternatively, Alexion commits to submit documentation to FDA demonstrating with due diligence that a suitable assay could not be feasibly developed and that the assessment of serum lactate dehydrogenase (LDH) is a sufficiently sensitive indicator of the presence of neutralizing antibodies. This information will be submitted to the BLA by July 9, 2008.

6. To utilize samples from the ongoing E05-001 Phase III extension study (approximately 170 patients for at least 2 years) as test samples for the new validated HAHA assays. Alexion will continue to obtain serum samples from those patients who transition from E05-001 to the Soliris Safety Registry, at intervals of no less than one year, and continue this collection process for an additional three years. Sample collection will cease during this additional three year period for patients who terminate eculizumab administration. Additionally, serum samples will be obtained based upon physician reports of suspected loss of eculizumab bioactivity, based upon unanticipated alterations in serum LDH concentrations. All serum samples will be assayed at least annually and the results provided within an annual report to the BLA. Clinical data, to include the results of serum LDH concentrations, will also be obtained from any patients who show evidence of antibody formation. The protocol describing Alexion's plan for responding to this commitment will be submitted by May 1, 2007 and the final study report submitted by January 31, 2011.

Postmarketing Studies not subject to reporting requirements of 21 CFR 601.70.

7. To revalidate the linearity and accuracy of the Osmolality method across the full specification range using a combination of product samples diluted to lower osmolality and product samples spiked with osmolality standards. The revalidation plan will be submitted in advance to FDA for review and endorsement. This information will be submitted to the BLA as a CBE 30 by August 31, 2007.

8. To revalidate the linearity of the IEF method across a load range of [—] The revalidation plan will be submitted in advance to FDA for review and endorsement. This information will be submitted to the BLA as a CBE 30 supplement by August 31, 2007.

9. To revise the IEF method SOP to specify that the method is validated only for a [— —] This information will be submitted to the BLA as a CBE 30 by August 31, 2007.

10. To improve and revalidate the existing hemolytic assay. Improvements include increasing the number of sample replicates and qualifying the chicken erythrocytes reagent. The revised method SOP and revalidation plan will be submitted in advance to FDA for review and endorsement. This information will be submitted to the BLA as a CBE 30 by August 31, 2007.

11. To develop a new quantitative biological activity assay to replace the existing hemolytic assay, or submit documentation to FDA demonstrating with due diligence that a suitable assay could not be feasibly developed. This information will be submitted to the BLA by February 29, 2008. Validation of the quantitative biological activity assay will be submitted by July 9, 2008.

12. To provide FDA with a completed drug substance and drug product container closure system leachables evaluation using end-of-shelf-life, long-term 2-8°C stability samples. This information will be submitted to the BLA as a CBE 30 by August 31, 2007.

13. To develop a suitable [redacted] assay and subsequently confirm [redacted] on three drug substance batches. This information will be submitted to the BLA as a CBE 30 by August 31, 2007.

We request that you submit clinical protocols to your IND, with a cross-reference letter to this biologics license application (BLA), STN BL 125166. Submit nonclinical and chemistry, manufacturing, and controls protocols and all study final reports to your BLA, STN BL 125166. Please use the following designators to label prominently all submissions, including supplements, relating to these postmarketing study commitments as appropriate:

- **Postmarketing Study Commitment Protocol**
- **Postmarketing Study Commitment Final Study Report**
- **Postmarketing Study Commitment Correspondence**
- **Annual Status Report of Postmarketing Commitment Studies**

For each postmarketing study subject to the reporting requirements of 21 CFR 601.70, you must describe the status in an annual report on postmarketing studies for this product. The status report for each study should include:

- information to identify and describe the postmarketing commitment,
- the original schedule for the commitment,
- the status of the commitment (i.e. pending, ongoing, delayed, terminated, or submitted),
- an explanation of the status including, for clinical studies, the patient accrual rate (i.e. number enrolled to date and the total planned enrollment), and
- a revised schedule if the study schedule has changed and an explanation of the basis for the revision.

As described in 21 CFR 601.70(e), we may publicly disclose information regarding these postmarketing studies on our Web site (http://www.fda.gov/cder/pmc/default.htm). Please refer to the February 2006 Guidance for Industry: Reports on the Status of Postmarketing Study Commitments - Implementation of Section 130 of the Food and Drug Administration Modernization Act of 1997 (see http://www.fda.gov/cder/guidance/5509fnl.htm) for further information.

Under 21 CFR Part 208, we have determined that this product poses a serious and significant public health concern requiring the distribution of a Medication Guide. Eculizumab is a product for which patient labeling could help prevent serious adverse effects and inform the patient of serious risks relative to benefit that could affect their decisions to use, or continue to use, the product. Therefore, a Medication Guide is necessary for safe and effective use of this product and FDA hereby approves the draft Medication Guide you submitted March 15, 2007. Please note that:

- this Medication Guide must be reprinted at the end of the package insert or accompany it [21 CFR 201.57(c)(18)];
- you are responsible for ensuring that this Medication Guide is available for distribution to every patient who is dispensed a prescription for this product [21 CFR 208];
- the final printed Medication Guide distributed to patients must conform to all conditions described in 21 CFR 208.24, including a minimum of 10 point text; and
- you are responsible for ensuring that the label of each container or package includes a prominent and conspicuous instruction to authorized dispensers to provide a Medication Guide to each patient to whom the drug is dispensed, and states how the Medication Guide is provided.

Please submit within 30 days content of labeling [21 CFR 601.14(b)] in structured product labeling (SPL) format, as described at http://www.fda.gov/oc/datacouncil/spl.html, that is identical in content to the enclosed labeling text dated March 16, 2007. Upon receipt and verification, we will transmit that version to the National Library of Medicine for public dissemination.

You must submit adverse experience reports under the adverse experience reporting requirements for licensed biological products (21 CFR 600.80). You should submit postmarketing adverse experience reports to the Central Document Room, Center for Drug Evaluation and Research, Food and Drug Administration, 5901-B Ammendale Road, Beltsville, MD 20705-1266. Prominently identify all adverse experience reports as described in 21 CFR 600.80.

The MedWatch-to-Manufacturer Program provides manufacturers with copies of serious adverse event reports that are received directly by the FDA. New molecular entities and important new biologics qualify for inclusion for three years after approval. Your firm is eligible to receive copies of reports for this product. To participate in the program, please see the enrollment instructions and program description details at www.fda.gov/medwatch/report/mmp.htm.

You must submit distribution reports under the distribution reporting requirements for licensed biological products (21 CFR 600.81).

You must submit reports of biological product deviations under 21 CFR 600.14. You should promptly identify and investigate all manufacturing deviations, including those associated with processing, testing, packing, labeling, storage, holding and distribution. If the deviation involves a distributed product, may affect the safety, purity, or potency of the product, and meets the other criteria in the regulation, you must submit a report on Form FDA-3486 to the Division of Compliance Risk Management and Surveillance (HFD-330), Center for Drug Evaluation and Research, Food and Drug Administration, 5600 Fishers Lane, Rockville, MD 20857. Biological product deviations sent by courier or overnight mail should be addressed to Food and Drug Administration, CDER, Office of Compliance, Division of Compliance Risk Management and Surveillance, HFD-330, Montrose Metro 2, 11919 Rockville Pike, Rockville, MD 20852.

Please submit all final printed labeling at the time of use and include implementation information on FDA Form 356h. Please provide a PDF-format electronic copy as well as original paper copies (ten for circulars and five for other labels). In addition, you may wish to submit draft copies of the proposed introductory advertising and promotional labeling with a cover letter requesting advisory comments to the Food and Drug Administration, Center for Drug Evaluation and Research, Division of Drug Marketing, Advertising and Communication, 5901-B Ammendale Road, Beltsville, MD 20705-1266. Final printed advertising and promotional labeling should be submitted at the time of initial dissemination, accompanied by a FDA Form 2253.

All promotional claims must be consistent with and not contrary to approved labeling. You should not make a comparative promotional claim or claim of superiority over other products unless you have substantial evidence to support that claim.

Please refer to http://www.fda.gov/cder/biologics/default.htm for information regarding therapeutic biological products, including the addresses for submissions.

Sincerely,

Karen Weiss for

Richard Pazdur, M.D.
Director
Office of Oncology Drug Products
Center for Drug Evaluation and Research

Enclosure: Package Insert
Carton and Vial Labeling
Medication Guide
Patient Safety Card

US006355245B1

(12) **United States Patent**
Evans et al.

(10) **Patent No.: US 6,355,245 B1**
(45) **Date of Patent: Mar. 12, 2002**

(54) **C5-SPECIFIC ANTIBODIES FOR THE TREATMENT OF INFLAMMATORY DISEASES**

(75) Inventors: **Mark J. Evans**, Cheshire; **Louis A. Matis**, Southport; **Eileen Elliott Mueller**, East Haven, all of CT (US); **Steven H. Nye**, Mequon, WI (US); **Scott Rollins**, Monroe, CT (US); **Russell P. Rother**; **Jeremy P. Springhorn**, both of Cheshire, CT (US); **Stephen P. Squinto**, Bethany, CT (US); **Thomas C. Thomas**, Madison, CT (US); **James A. Wilkins**, Woodbridge, CT (US)

(73) Assignee: **Alexion Pharmaceuticals, Inc.**, Cheshire, CT (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **08/487,283**

(22) Filed: **Jun. 7, 1995**

Related U.S. Application Data

(63) Continuation of application No. PCT/US95/05688, filed on May 1, 1995, which is a continuation-in-part of application No. 08/236,208, filed on May 2, 1994, now Pat. No. 6,074,642.

(51) **Int. Cl.**[7] **A61K 39/395**; C07K 16/36; C12N 5/12

(52) **U.S. Cl.** ..... **424/145.1**; 424/130.1; 424/133.1; 424/135.1; 424/141.1; 424/145.1; 424/158.1; 424/139.1; 424/130; 530/387.1; 530/387.3; 530/387.9; 530/388.1; 530/388.23; 530/388.25; 530/388.7; 435/326; 435/328; 435/331; 435/33 L; 435/337; 435/343; 435/346

(58) **Field of Search** 530/387.1, 387.3, 530/388.1, 388.7, 387.9, 388.23; 435/69.1, 172.3, 328, 343, 70.21, 325, 331, 332, 346; 424/130.1, 141.1, 139, 133.1, 145.1

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(List continued on next page.)

Primary Examiner—Phillip Gambel

(57) **ABSTRACT**

The use of anti-C5 antibodies, e.g., monoclonal antibodies, to treat glomerulonephritis (GN) is disclosed. The administration of such antibodies at low dosage levels has been found to significantly reduce glomerular inflammation/enlargement and other pathologic conditions associated with GN. Also disclosed are anti-C5 antibodies and anti-C5 antibody-encoding nucleic acid molecules. These antibodies are useful in the treatment of GN and other inflammatory conditions involving pathologic activation of the complement system.

23 Claims, 19 Drawing Sheets

Exhibit C

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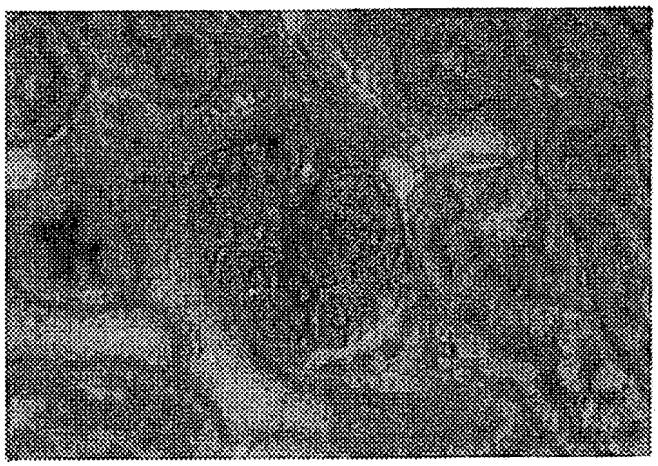

FIG. 1A

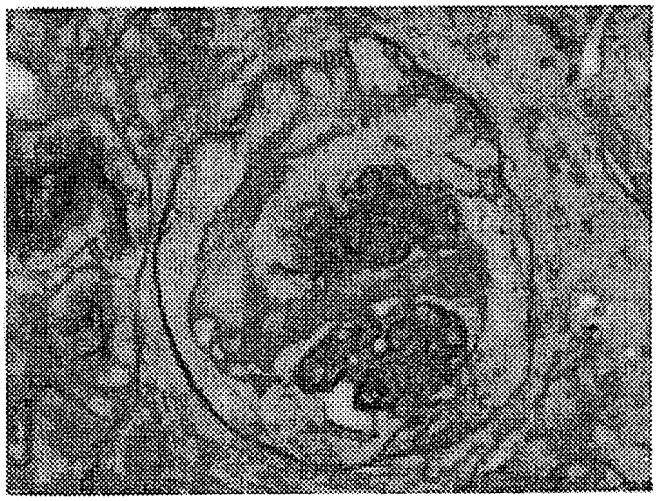

FIG. 1B

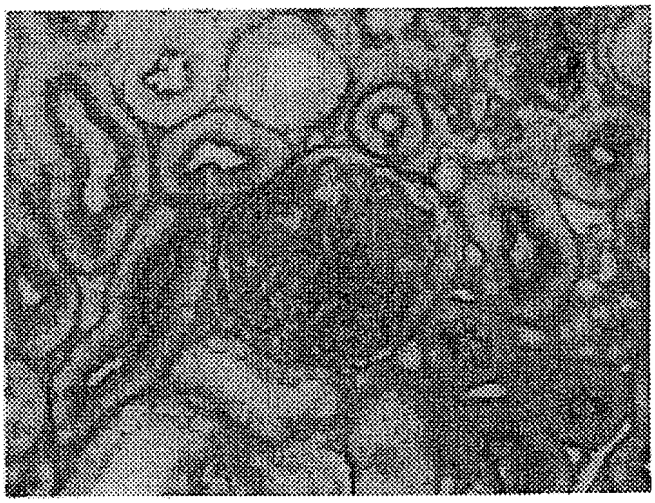

FIG. 1C

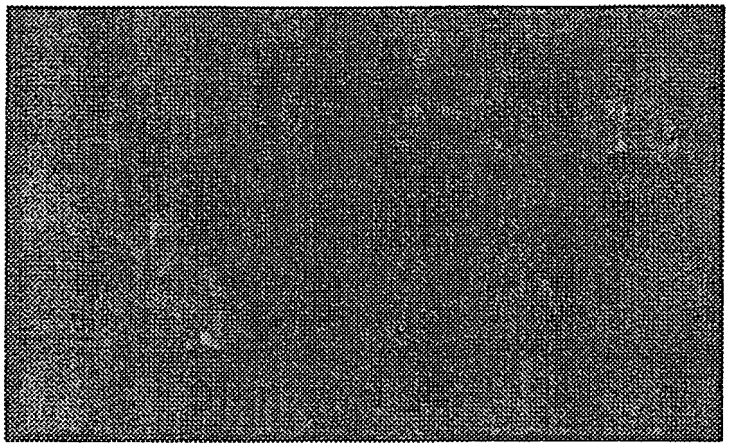

FIG. 2A

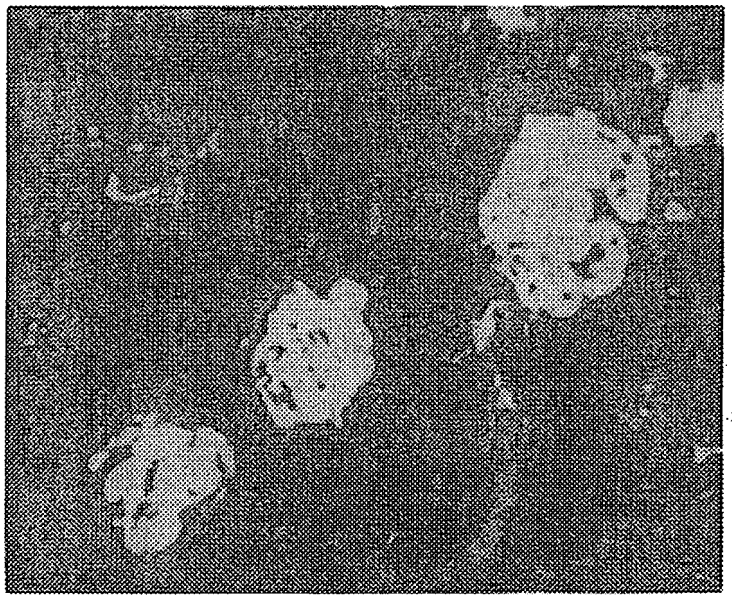

FIG. 2B

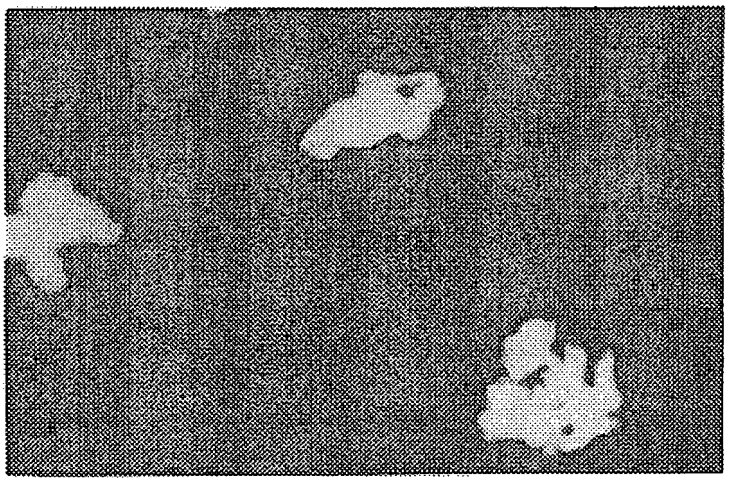

FIG. 2C

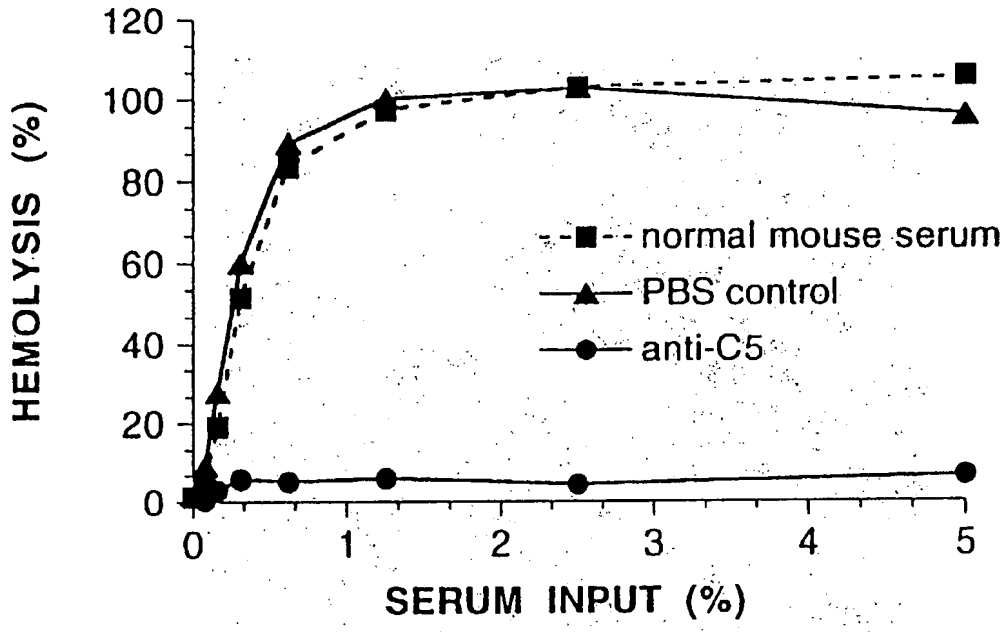
HEMOLYSIS (%)
120
100
80
60
40
20
0
0
1
2
3
4
5
SERUM INPUT (%)
normal mouse serum
PBS control
anti-C5

FIG.3

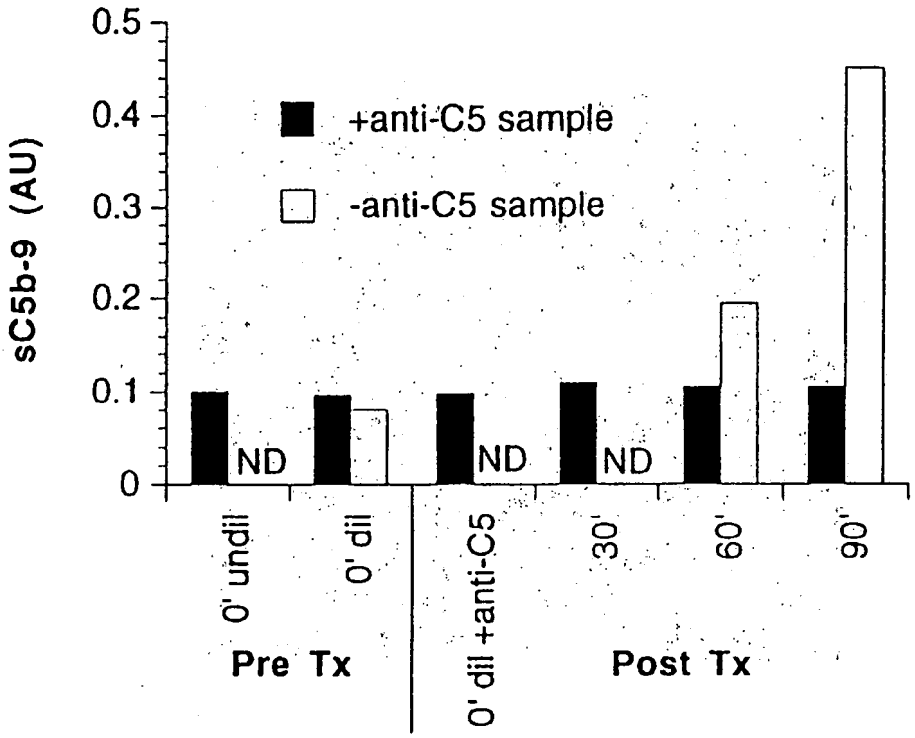
0.5
0.4
0.3
0.2
0.1
0
sC5b-9 (AU)
+anti-C5 sample
-anti-C5 sample
ND
ND
ND
0' undil
0' dil
0' dil +anti-C5
30'
60'
90'
Pre Tx
Post Tx

FIG.4

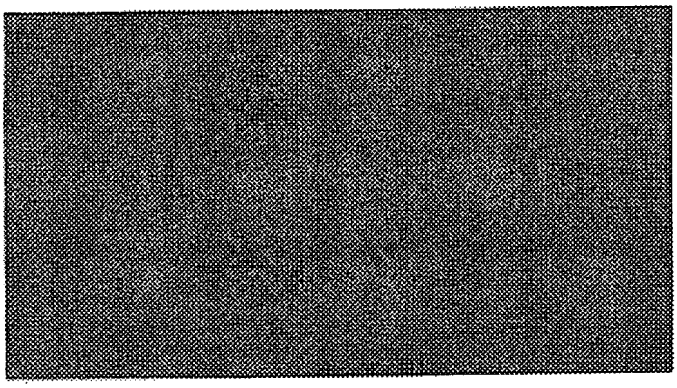

FIG.5A

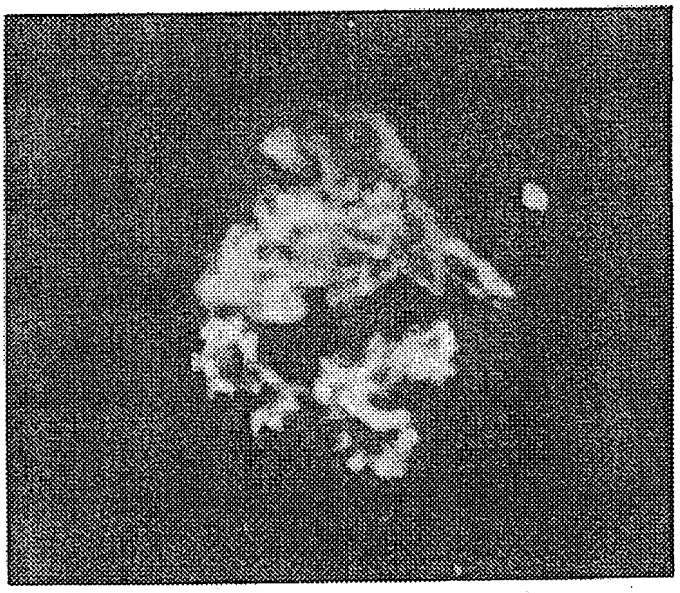

FIG.5B

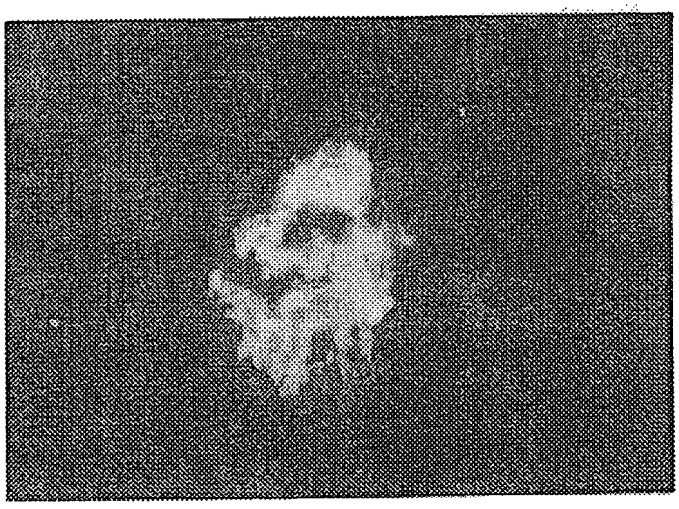

FIG.5C

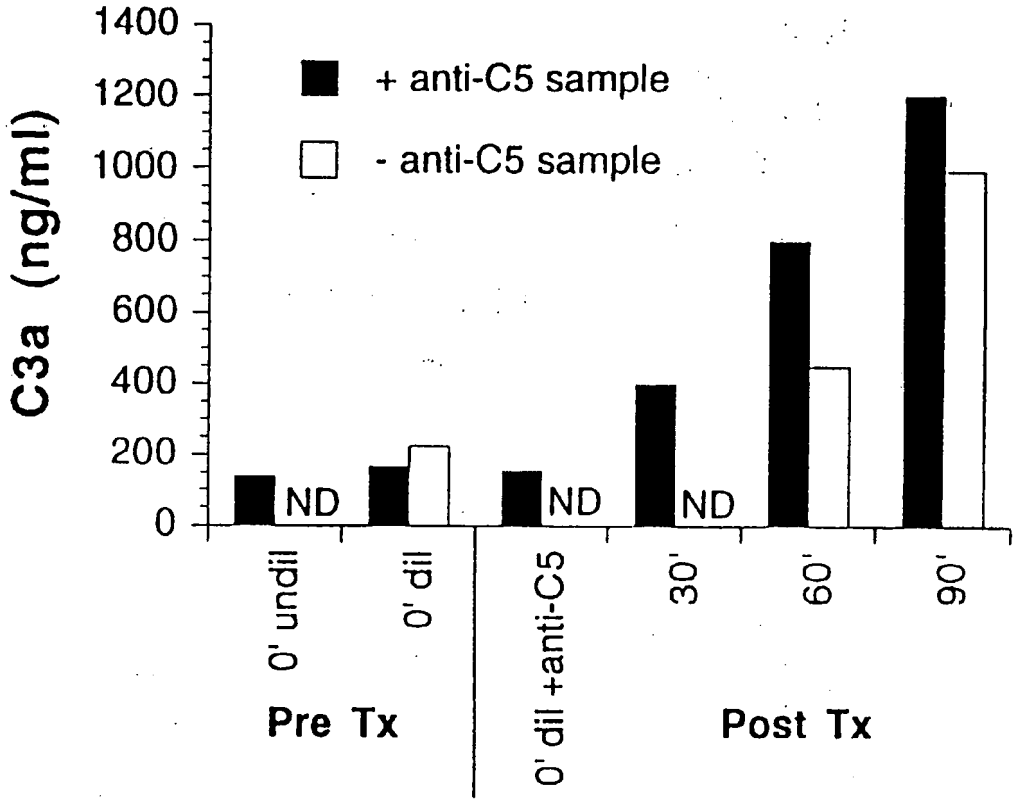
1400
1200
1000
800
600
400
200
0
C3a (ng/ml)
+ anti-C5 sample
- anti-C5 sample
ND
ND
ND
0' undil
0' dil
0' dil +anti-C5
30'
60'
90'
Pre Tx
Post Tx

FIG.6

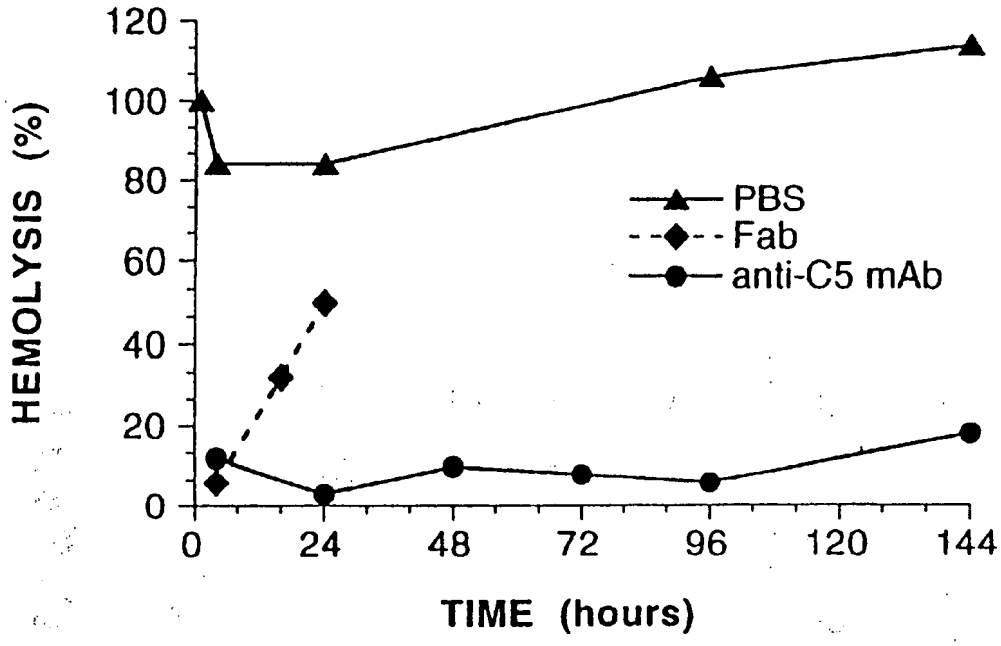
HEMOLYSIS (%)
120
100
80
60
40
20
0
0
24
48
72
96
120
144
TIME (hours)
PBS
Fab
anti-C5 mAb

FIG.7A

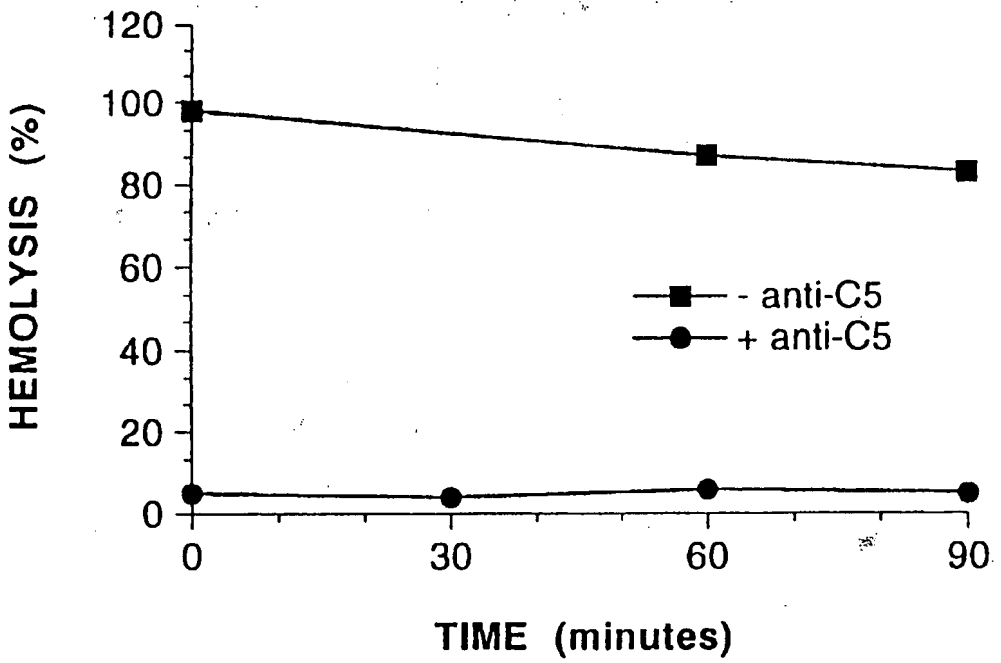
HEMOLYSIS (%)
120
100
80
60
40
20
0
0
30
60
90
TIME (minutes)
- anti-C5
+ anti-C5

FIG.7B

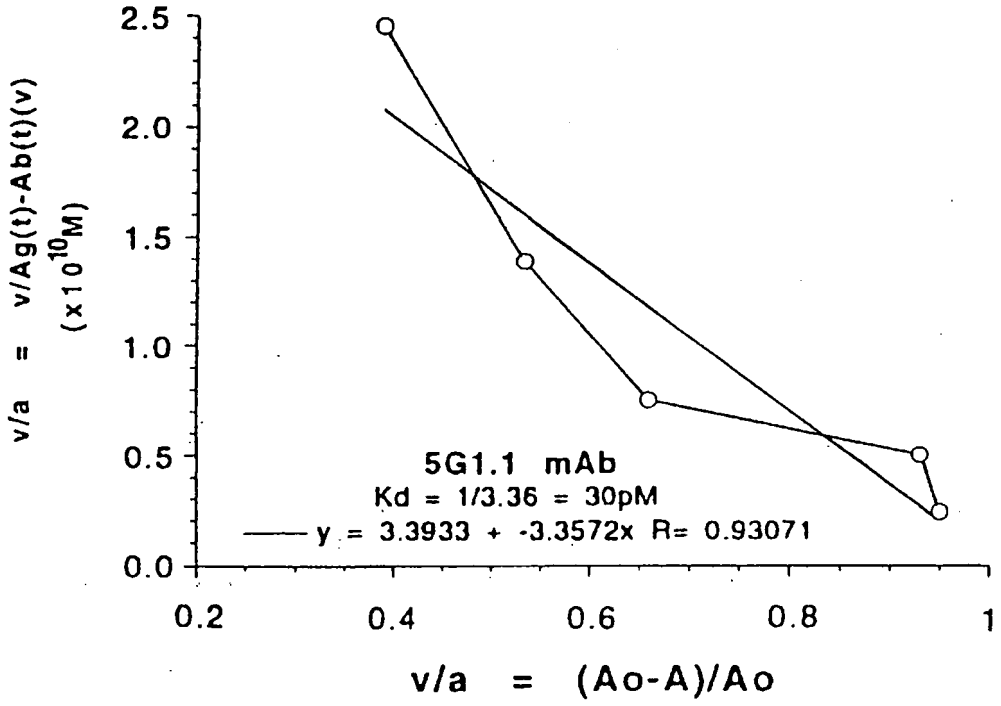
v/a = v/Ag(t)-Ab(t)(v)
(x10^10M)
2.5
2.0
1.5
1.0
0.5
0.0
0.2
0.4
0.6
0.8
1
5G1.1 mAb
Kd = 1/3.36 = 30pM
y = 3.3933 + -3.3572x R= 0.93071
v/a = (Ao-A)/Ao

FIG.8

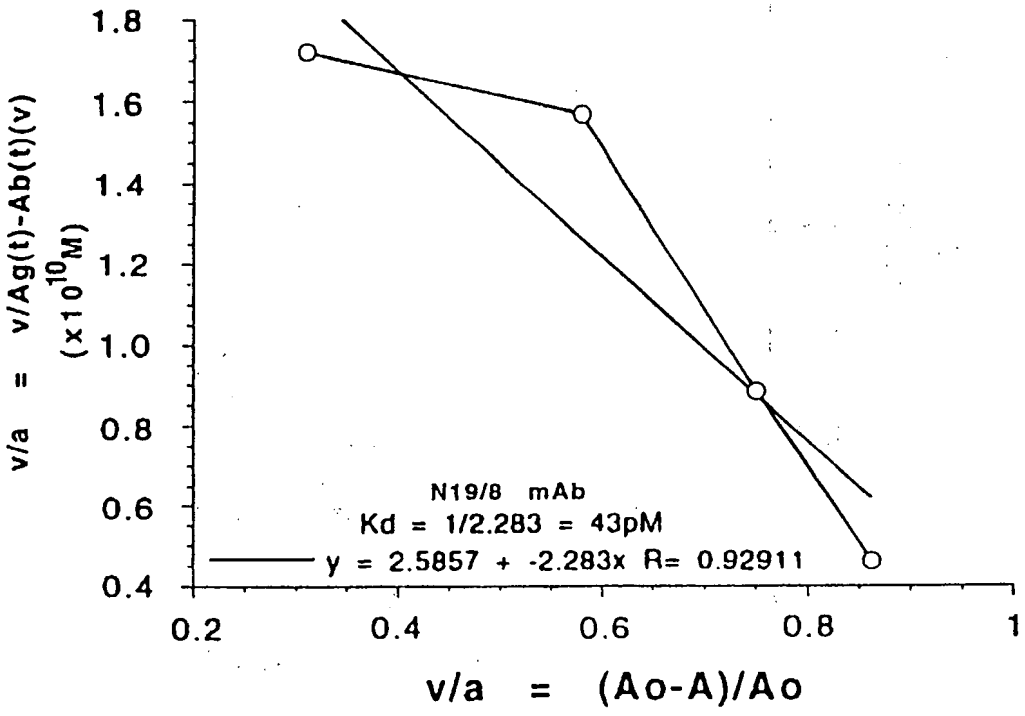
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
v/a = v/Ag(t)-Ab(t)(v)
(x10^10M)
N19/8 mAb
Kd = 1/2.283 = 43pM
y = 2.5857 + -2.283x R= 0.92911
0.2
0.4
0.6
0.8
1
v/a = (Ao-A)/Ao

FIG.9

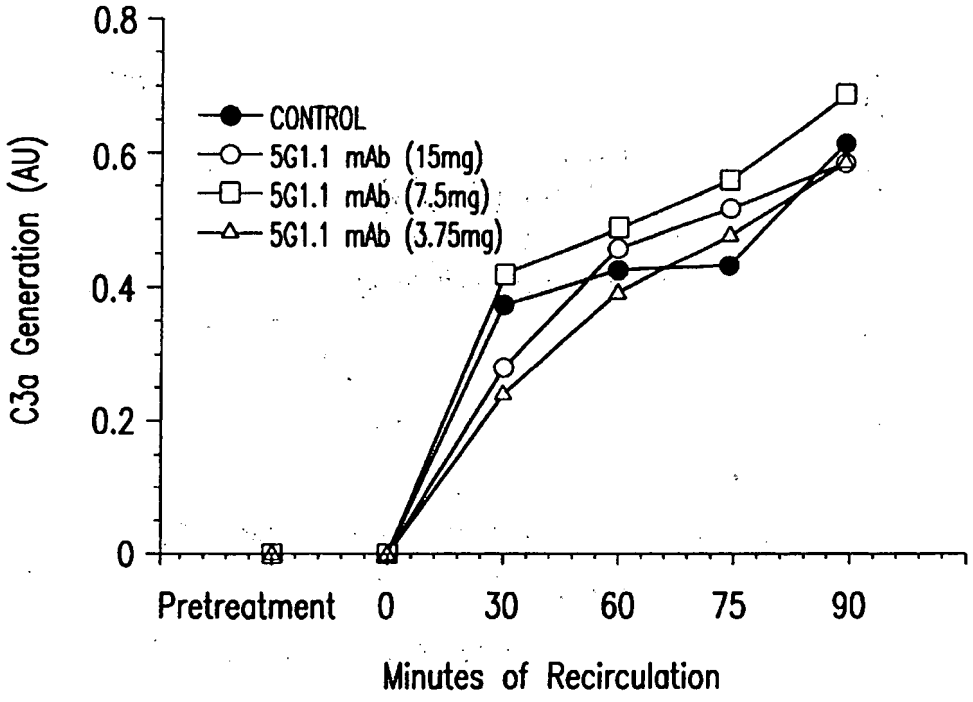
0.8
0.6
0.4
0.2
0
C3a Generation (AU)
CONTROL
5G1.1 mAb (15mg)
5G1.1 mAb (7.5mg)
5G1.1 mAb (3.75mg)
Pretreatment
0
30
60
75
90
Minutes of Recirculation

FIG.10

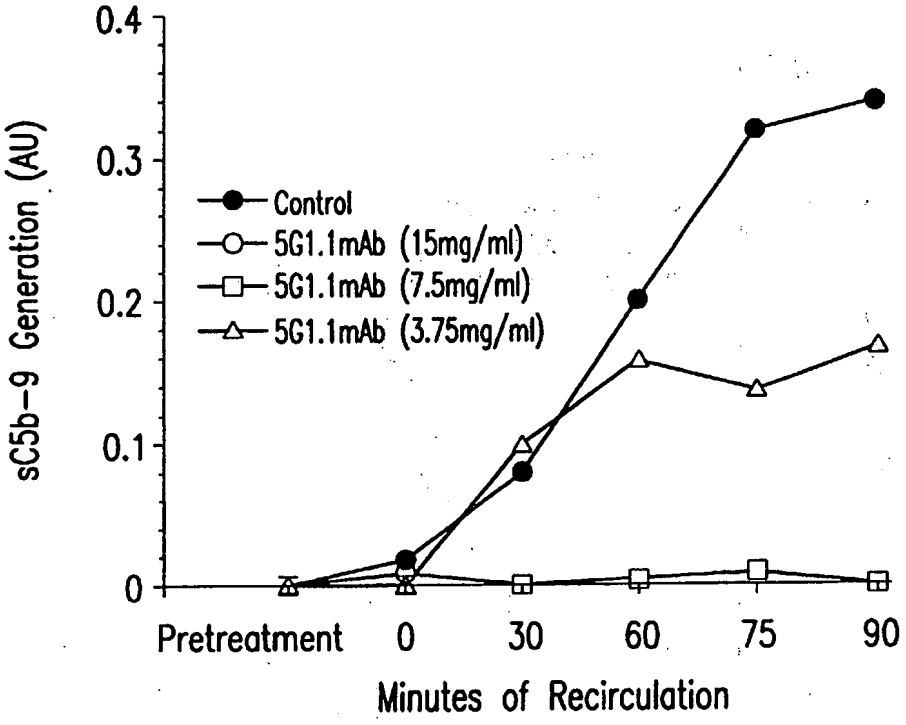
0.4
0.3
0.2
0.1
0
sC5b-9 Generation (AU)
Control
5G1.1mAb (15mg/ml)
5G1.1mAb (7.5mg/ml)
5G1.1mAb (3.75mg/ml)
Pretreatment
0
30
60
75
90
Minutes of Recirculation

FIG. 11

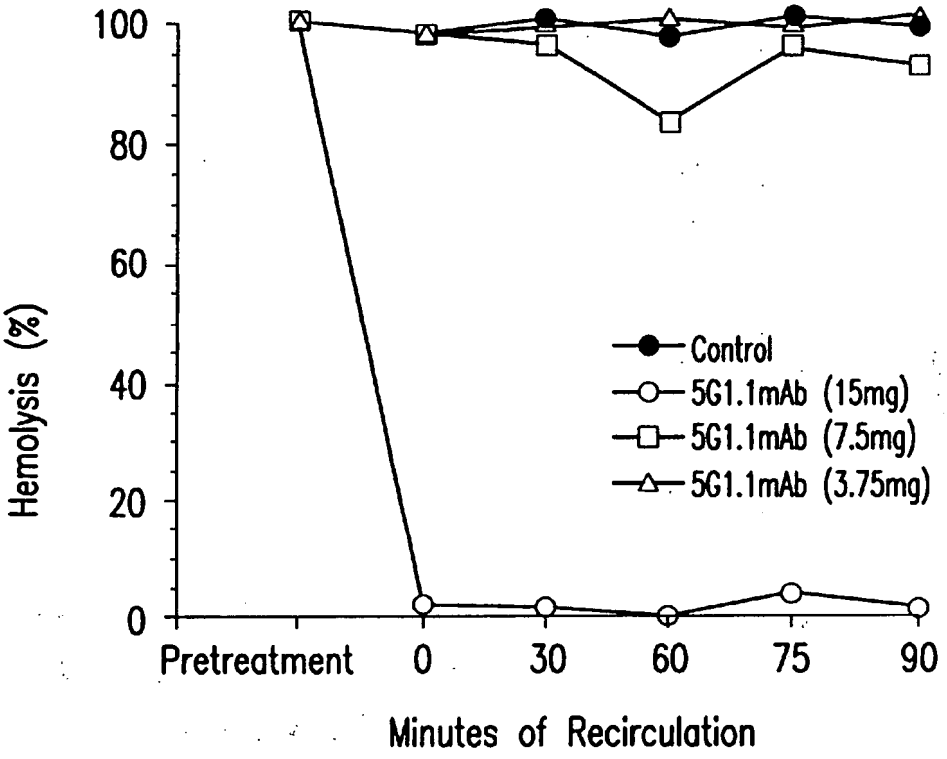
100
80
60
40
20
0
Hemolysis (%)
Control
5G1.1mAb (15mg)
5G1.1mAb (7.5mg)
5G1.1mAb (3.75mg)
Pretreatment
0
30
60
75
90
Minutes of Recirculation

FIG. 12

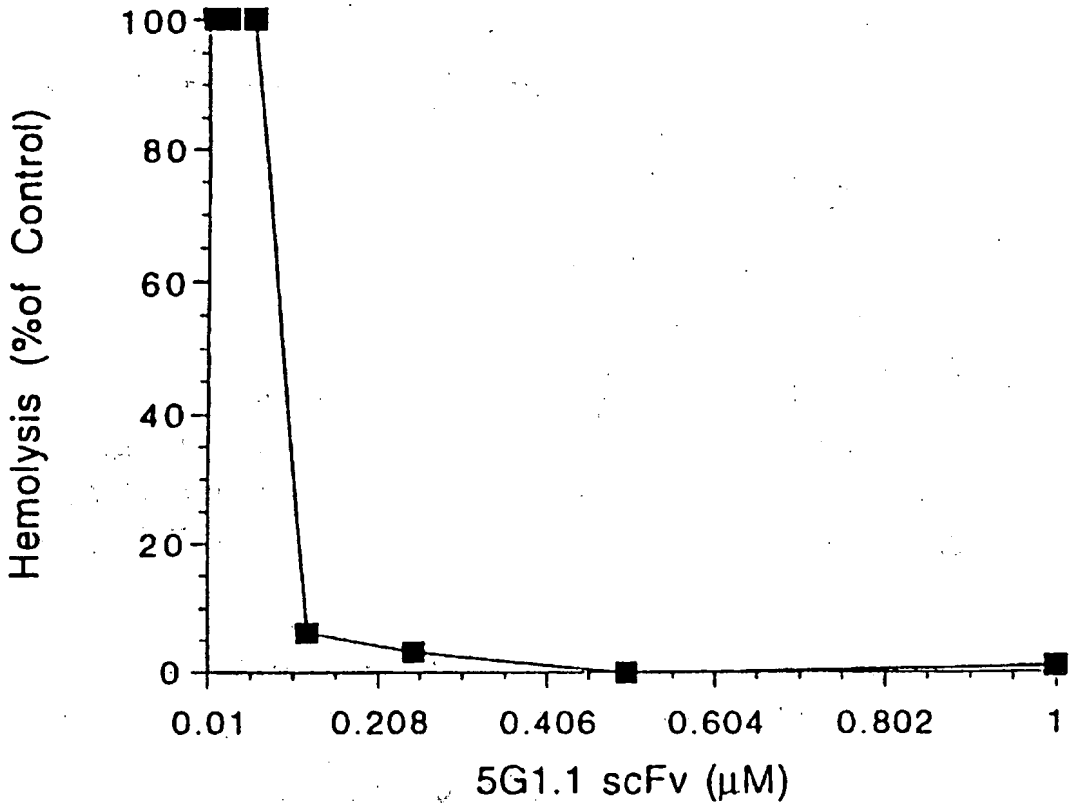
100
80
60
40
20
0
Hemolysis (%of Control)
0.01
0.208
0.406
0.604
0.802
1
5G1.1 scFv (μM)

FIG.13

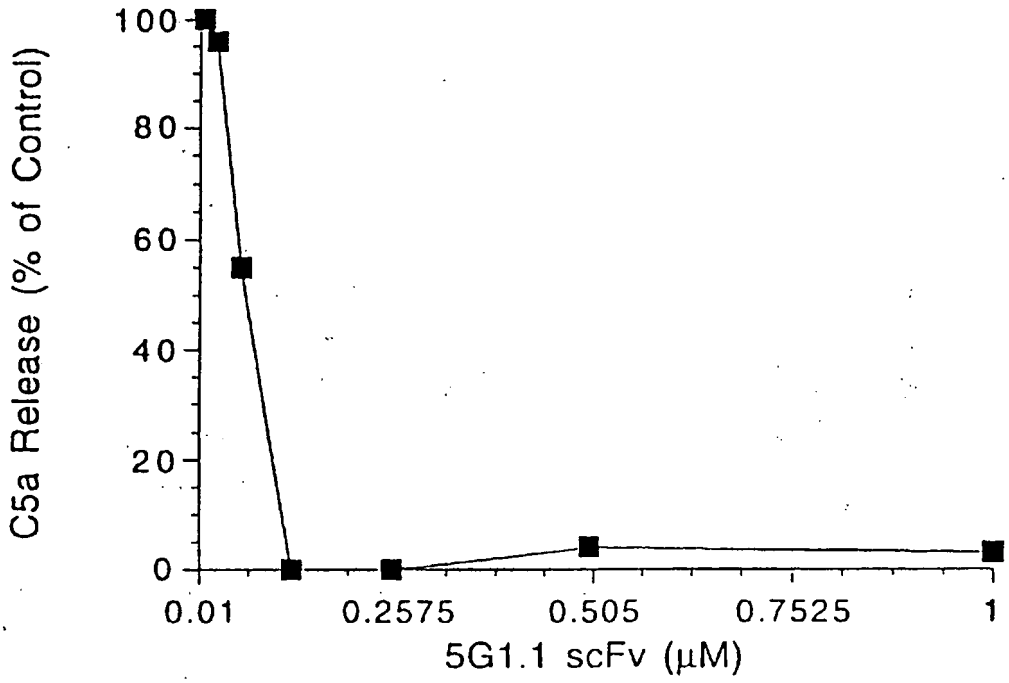
C5a Release (% of Control)
100
80
60
40
20
0
0.01
0.2575
0.505
0.7525
1
5G1.1 scFv (μM)

FIG.14

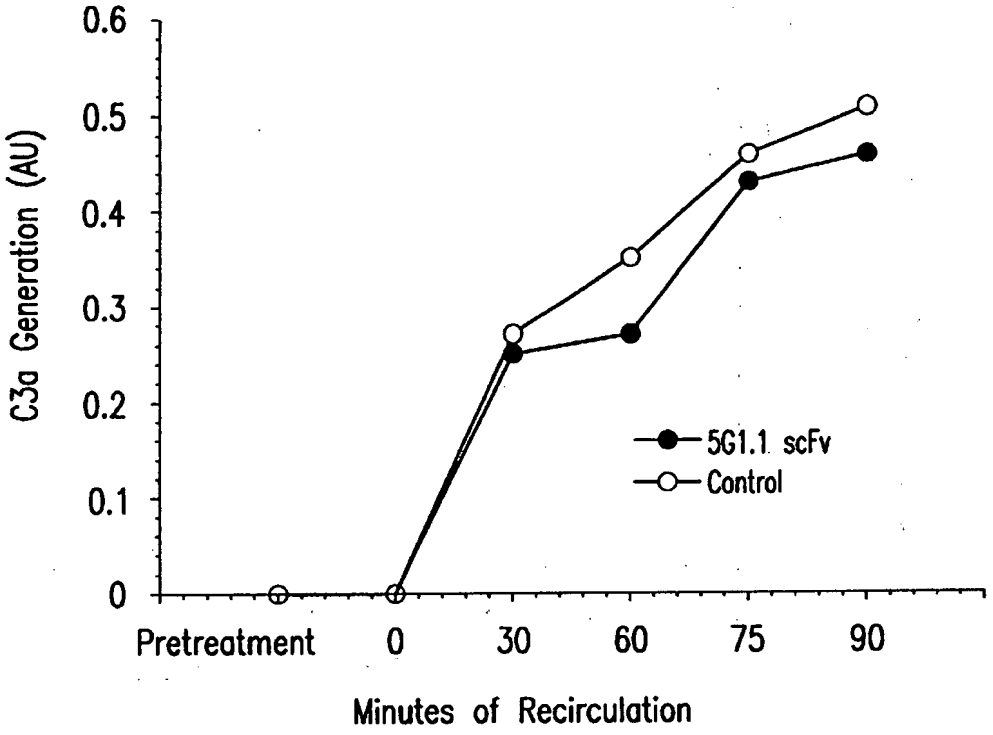
0.6
0.5
0.4
0.3
0.2
0.1
0
C3a Generation (AU)
5G1.1 scFv
Control
Pretreatment
0
30
60
75
90
Minutes of Recirculation

FIG. 15

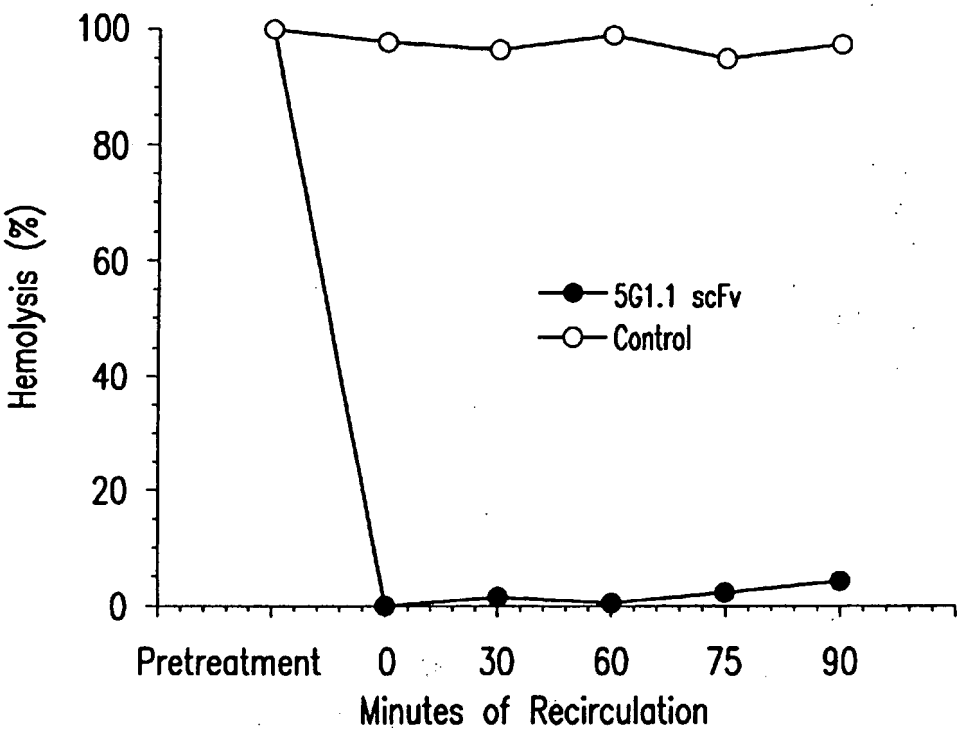
100
80
60
40
20
0
Hemolysis (%)
5G1.1 scFv
Control
Pretreatment
0
30
60
75
90
Minutes of Recirculation

FIG. 16

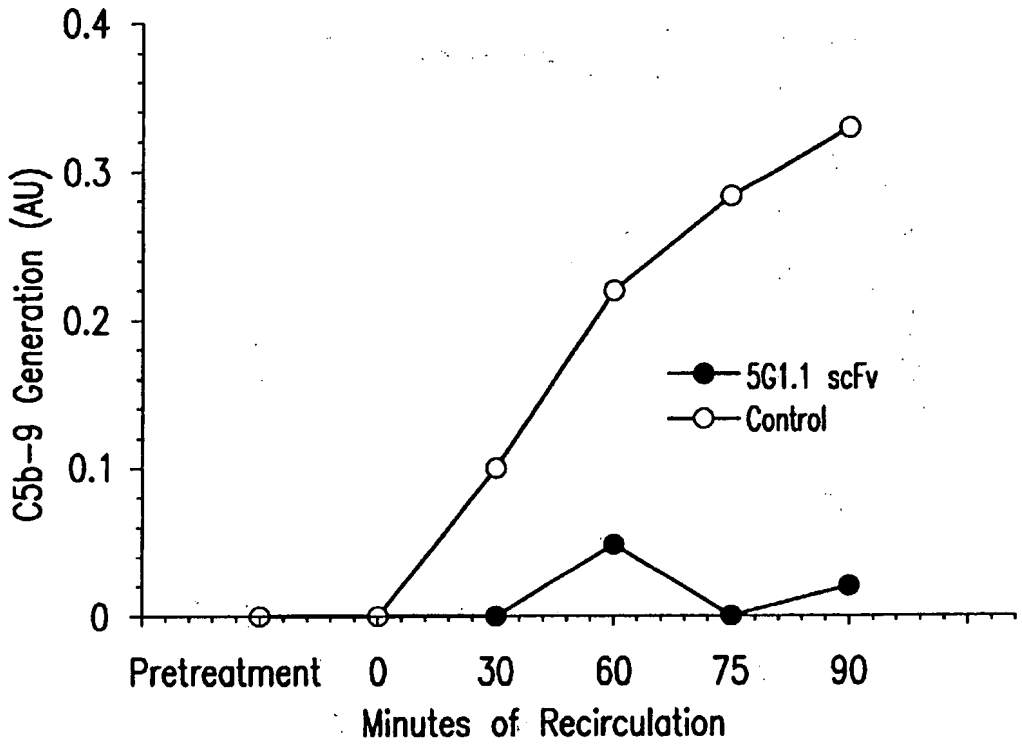
0.4
0.3
0.2
0.1
0
C5b-9 Generation (AU)
5G1.1 scFv
Control
Pretreatment
0
30
60
75
90
Minutes of Recirculation

FIG. 17

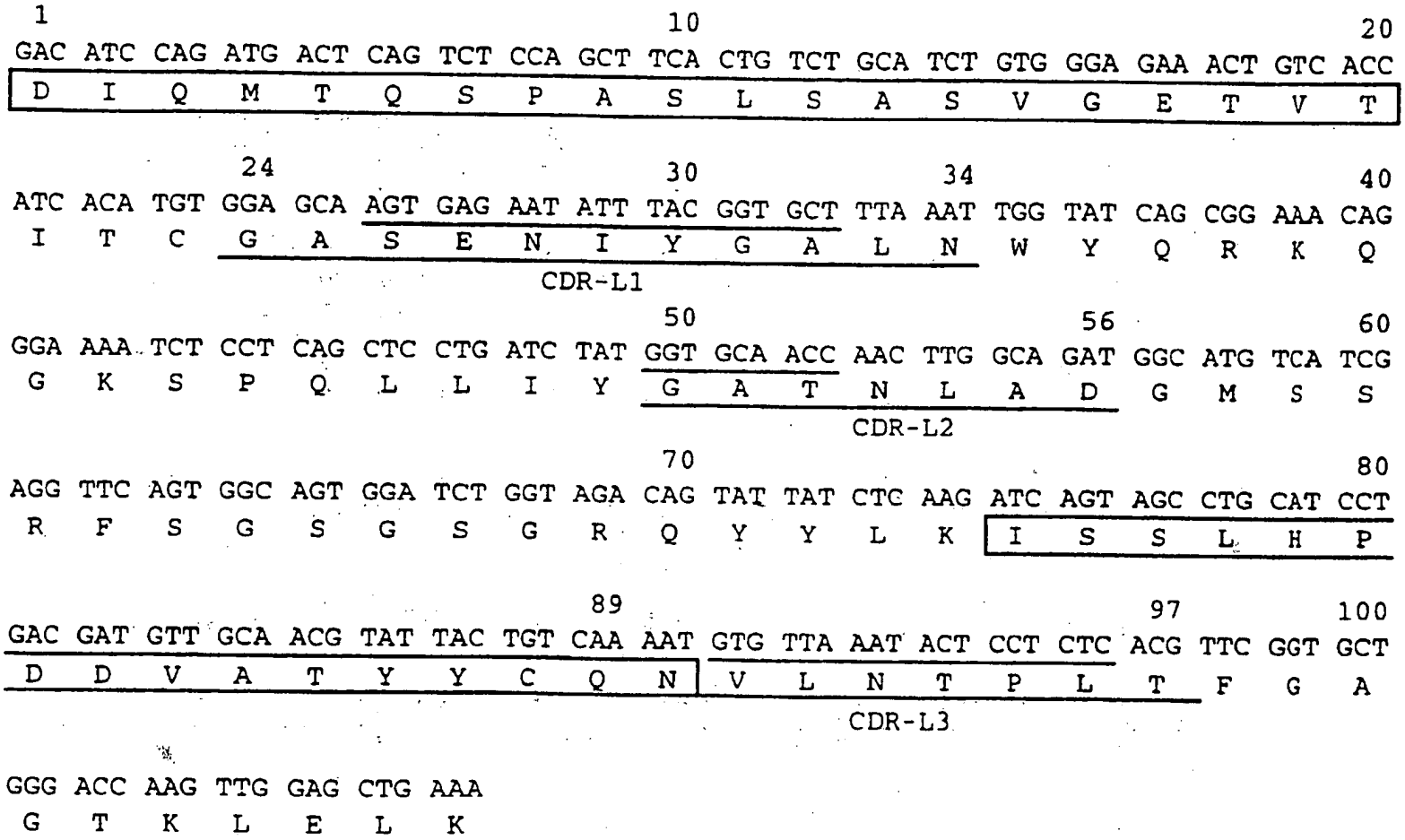
1
10
20
GAC ATC CAG ATG ACT CAG TCT CCA GCT TCA CTG TCT GCA TCT GTG GGA GAA ACT GTC ACC
D I Q M T Q S P A S L S A S V G E T V T
24
30
34
40
ATC ACA TGT GGA GCA AGT GAG AAT ATT TAC GGT GCT TTA AAT TGG TAT CAG CGG AAA CAG
I T C G A S E N I Y G A L N W Y Q R K Q
CDR-L1
50
56
60
GGA AAA TCT CCT CAG CTC CTG ATC TAT GGT GCA ACC AAC TTG GCA GAT GGC ATG TCA TCG
G K S P Q L L I Y G A T N L A D G M S S
CDR-L2
70
80
AGG TTC AGT GGC AGT GGA TCT GGT AGA CAG TAT TAT CTC AAG ATC AGT AGC CTG CAT CCT
R F S G S G S G R Q Y Y L K I S S L H P
89
97
100
GAC GAT GTT GCA ACG TAT TAC TGT CAA AAT GTG TTA AAT ACT CCT CTC ACG TTC GGT GCT
D D V A T Y Y C Q N V L N T P L T F G A
CDR-L3
GGG ACC AAG TTG GAG CTG AAA
G T K L E L K

FIG.18

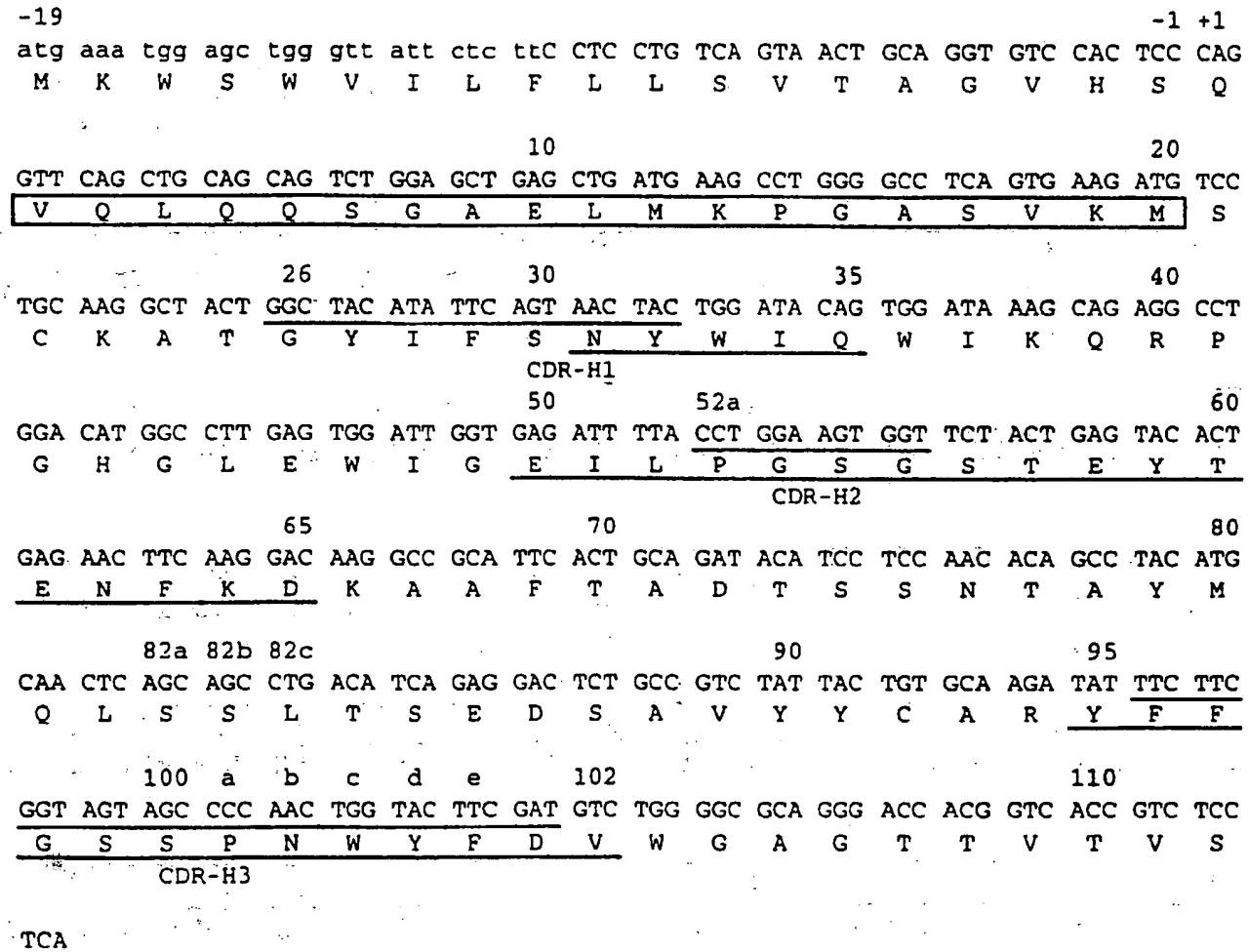
-19 -1 +1
atg aaa tgg agc tgg gtt att ctc ttC CTC CTG TCA GTA ACT GCA GGT GTC CAC TCC CAG
M K W S W V I L F L L S V T A G V H S Q
10 20
GTT CAG CTG CAG CAG TCT GGA GCT GAG CTG ATG AAG CCT GGG GCC TCA GTG AAG ATG TCC
V Q L Q Q S G A E L M K P G A S V K M S
26 30 35 40
TGC AAG GCT ACT GGC TAC ATA TTC AGT AAC TAC TGG ATA CAG TGG ATA AAG CAG AGG CCT
C K A T G Y I F S N Y W I Q W I K Q R P
CDR-H1
50 52a 60
GGA CAT GGC CTT GAG TGG ATT GGT GAG ATT TTA CCT GGA AGT GGT TCT ACT GAG TAC ACT
G H G L E W I G E I L P G S G S T E Y T
CDR-H2
65 70 80
GAG AAC TTC AAG GAC AAG GCC GCA TTC ACT GCA GAT ACA TCC TCC AAC ACA GCC TAC ATG
E N F K D K A A F T A D T S S N T A Y M
82a 82b 82c 90 95
CAA CTC AGC AGC CTG ACA TCA GAG GAC TCT GCC GTC TAT TAC TGT GCA AGA TAT TTC TTC
Q L S S L T S E D S A V Y Y C A R Y F F
100 a b c d e 102 110
GGT AGT AGC CCC AAC TGG TAC TTC GAT GTC TGG GGC GCA GGG ACC ACG GTC ACC GTC TCC
G S S P N W Y F D V W G A G T T V T V S
CDR-H3
TCA
S

FIG.19

C5-SPECIFIC ANTIBODIES FOR THE TREATMENT OF INFLAMMATORY DISEASES

This is a continuation in part of International application Ser. No. PCT/US95/05688, filed May 1, 1995, which is a continuation in part of U.S. application Ser. No. 08/236,208, filed May 2, 1994, which issued as U.S. Pat. No. 6,074,642 on Jun. 13, 2000. International application Serial No. PCT/US95/05688 was published in English under PCT Article 21(2) on Nov. 9, 1995 as WO 95/29697.

FIELD OF THE INVENTION

The present invention relates to the treatment of glomerulonephritis (GN) and other inflammatory diseases, and more generally to therapeutic treatments involving the pharmacologic inhibition of a patient's complement system. In particular, the invention relates to the use of antibodies specific to human complement component C5 to accomplish such therapeutic treatment. The invention also relates to compositions comprising native monoclonal antibodies (mAbs) specific to human complement component C5 that block complement hemolytic activity and C5a generation at concentrations that substantially reach the theoretical one to two stoichiometric limit of antibody to antigen that can be achieved by a bivalent antibody. The invention further provides recombinant mabs that are derivatives (including monovalent derivatives) of these native mAbs that provide substantially the same blocking activities as the native mabs.

BACKGROUND OF THE INVENTION

I. Immune Complex Mediated Disease

The formation of immune complexes is the typical consequence of the interaction of antigens with specific antibodies. The inflammatory response that ensues when such complexes accumulate in a limited area is an important element of normal host defenses, leading to immune complex clearance and antigen destruction by phagocytic cells. In contrast, immune complex diseases are reflections of excess complex formation or retarded clearance, usually under conditions of exceptional antigen challenge or immunologic dysregulation. Under such circumstances, immune complexes are deposited or formed at specific tissue sites and resulting inflammatory responses lead to disease states due to localized or systemic tissue damage. The kidney, and more specifically the kidney structure known as the glomerulus, is a particularly important site of immune complex deposition resulting in the development of serious disease conditions.

Human studies, and studies using animal models of human diseases, have implicated the complement system in the pathologies associated with a number of immune complex associated disorders. The activation of complement that mediates the pathology associated with these disorders may be a consequence of an autoimmune mechanism, or can be non-immunologic in origin.

The hypersensitivity response that occurs when antibodies bind to antigens either in tissues or in the circulation results from the activation of complement and the release of molecules that mediate inflammation. This process is classified as either being mediated by the binding of antibody to fixed tissue or cell bound antigens (Type II hypersensitivity) or to circulating antigens, resulting in the formation of circulating immune complexes and their subsequent pathogenic deposition in tissues (Type III hypersensitivity).

Type II hypersensitivity is mediated through the activation of complement following the binding of antibodies to fixed tissue antigens. The inflammatory response that ensues results from the activation of the proinflammatory and lytic components of the complement system and the subsequent recruitment of stimulated leukocytes to the sites of immune complex formation. The increased vascular permeability that results from the anaphylatoxic activities of C3a and C5a further enhances immune complex deposition and leukocyte recruitment.

The cross-linking of antibody bound cells or tissues to effector cells such as neutrophils, platelets, NK cells, and monocytes via their Fc receptors also plays a proinflammatory role. Such cross-linking activates effector cells, stimulating the release of oxygen radicals, prostaglandins, and leukotrienes, which release is further potentiated by the actions of activated complement components.

Examples of Type II hypersensitivity-mediated conditions include hyperacute rejection of transplanted organs, autoimmune hemolytic and thrombocytopenic states, Goodpasture's syndrome (and associated glomerulonephritis and pulmonary hemorrhage), myasthenia gravis, pathologic sequellae associated with insulin-dependent diabetes melitus, and pemphigus vulgaris.

Type III hypersensitivity reactions involving circulating antigens can also result in the development of numerous pathologic conditions. These include glomerulonephritis (discussed in detail below), vasculitis (a potentially life-threatening inflammatory condition of large and/or small blood vessels), rheumatoid arthritis, dermatitis, and other disorders.

Other diseases associated with type III hypersensitivity reactions include autoimmune diseases such as systemic lupus erythematosis (SLE), many infectious diseases, neoplastic diseases, and a wide variety of other conditions (Dixon, et al. *Immune Complex Injury*, in Samter, (ed.) Immunological Diseases, 4th ed. Little Brown & Co. Boston, 1987).

II. Glomerulonephritis

The glomerulus is a key structural and functional element of the kidney. Each glomerulus is found as part of a larger structure that serves as the main functional unit of the kidney and is called a nephron. About a million nephrons are found in each kidney. Each glomerulus is a network of up to fifty parallel capillaries encased in a structure known as Bowman's capsule. The area inside Bowman's capsule that is not taken up by the glomerular capillaries is known as Bowman's space. The glomerulus functions as a filter, separating water and certain solutes from the proteins and cells of the blood into Bowman's space for further processing in the convoluted tubules, loop of Henle, and collecting duct of the nephron.

Glomerulonephritis (GN) is a disease of the kidney characterized by inflammation and resulting enlargement of the glomeruli that is typically due to immune complex formation. The accumulation of immune complexes in the glomeruli results in inflammatory responses, involving inter alia hypercellularity, that can cause total or partial blockage of the glomerulus through, among other factors, narrowing of capillary lumens. One result of this process is the inhibition of the normal filtration function of the glomerulus. Blockage may occur in large numbers of glomeruli, directly compromising kidney function and often causing the abnormal deposition of proteins in the walls of the capillaries making up the glomerulus. Such deposition can, in turn, cause damage to glomerular basement membranes. Those glomeruli that are not blocked develop increased permeability, allowing large amounts of protein to pass into the urine, a condition referred to as proteinuria.

In many cases of severe GN, pathological structures called crescents are formed within the Bowman's space, further impeding glomerular filtration. These structures can only be seen by microscopic examination of tissue samples obtained by biopsy or necropsy, and are thus not always observed in those patients in which they occur. Crescents are a manifestation of hypercellularity and are thought to arise from the extensive abnormal proliferation of parietal epithelial cells, the cells that form the inner lining of the Bowman's capsule. Clinical research has shown that there is a rough correlation between the percentage of glomeruli with crescents and the clinical severity of the disease, and thus the patient's prognosis. When present in large numbers, crescents are a poor prognostic sign.

Symptoms of GN include: proteinuria; reduced glomerular filtration rate (GFR); serum electrolyte changes including azotemia (uremia, excessive blood urea nitrogen—BUN) and salt retention, leading to water retention resulting in hypertension and edema; hematuria and abnormal urinary sediments including red cell casts; hypoalbuminemia; hyperlipidemia; and lipiduria.

In 1990, over 210,000 patients in the United States required hemodialysis or transplantation for chronic renal failure at an annual cost in excess of 7 billion dollars, according to the United States Renal Data System (USRDS). The USRDS compiles data on kidney disease in the United States in conjunction with the National Institute of Diabetes and Digestive and Kidney Diseases, Division of Kidney, Urologic, and Hematologic Diseases, of the National Institutes of Health (NIDDKD). The USRDS estimates that the costs of treatment for renal failure are now increasing by 20 percent annually.

GN is the third leading cause of death in end-stage renal disease patients, exceeded only by diabetes and hypertension. As a result, there is a clear and long felt need in the medical community for effective treatments for this condition. Research aimed at the development of new treatments for GN is ongoing worldwide. In the United States, the NIDDKD, the National Kidney Foundation, and several other public and private organizations sponsor research in this area. The National Kidney Foundation alone supplies over two million dollars annually to fund the efforts of kidney researchers.

III. Current Treatments for GN

Corticosteroid administration, typically as high doses of "pulse" intravenous methylprednisolone or oral prednisone therapy, is currently considered the most effective pharmacologic agent available for the treatment of GN. Such steroid therapy is often administered in combination with cytotoxic general immunosuppressive agents such as azathioprine or cyclophosphamide. The overall immune suppression and resulting increased susceptibility to infection, along with other debilitating side effects associated with both steroid and cytotoxic drug administration, limit the effective use of these drugs.

Aspirin-like non-steroidal anti-inflammatory drugs (NSAIDs) have also been used to reduce the glomerular inflammation and enlargement of GN. These drugs are not routinely used for this purpose, however, probably because of their relatively weak anti-inflammatory effects and propensity to cause gastrointestinal and other side effects in many patients.

The administration of anticoagulants such as heparin or warfarin sodium, and antithrombotic agents such as cyproheptadine, dipyridamole, or sulfinpyrazone, has been used on the basis of evidence suggesting the involvement of the coagulation process in the genesis of glomerular crescents. However, objective evidence of benefit from such therapies in animals afflicted with experimentally induced crescentic GN has been inconsistent. Also, anticoagulants are dangerous drugs, as they can potentiate life-threatening bleeding episodes. They are especially hazardous in this regard in patients with advanced renal failure.

In addition to pharmacologic approaches, intensive plasma exchange (plasmapheresis) of 2 to 4 liters of plasma daily (or in some cases three times a week) can dramatically reduce high levels of circulating immune complexes when acute intervention in the inflammatory process is needed. Such treatment is expensive and requires that the patient be connected to the plasmapheresis machine for many hours each week. In addition, all procedures in which blood is removed from and returned to a patient are associated with an increased risk of infection. Nonetheless, plasma exchange is currently considered the most effective non-pharmacological treatment for removal of circulating immune complexes which can cause GN.

Circulating immune complex levels can also be decreased by eliminating or reducing the source of the antigen or antigens contained in the complexes by, for example, effective therapy of an underlying infection or change in an antibiotic. However, while such therapy is almost always a treatment of choice, great care must be taken since reduction of the antigen load alters the molar ratio of antigen to antibody involved in forming immune complexes and thus a dangerous temporary exacerbation of the inflammatory process may occur (see discussion below in Background Physiology & Pathology).

IV. Antibody Engineering

Native antibodies are multi-subunit animal protein molecules with highly specific antigen-binding properties. Animals make multiple classes of antibodies. There are five major classes (IgA, IgD, IgE, IgG and IgM) and a variety of subclasses. Native antibodies are made up of two or more heterodimeric subunits each containing one heavy (H) and one light (L) chain. The differences between antibody classes derive from their different H chains. H chains have a molecular weight of about 53 kDa, while L chains are about 23 kDa in mass.

Every individual native antibody has one type of L chain and one type of H chain, which are held together by disulfide bonds to form a heterodimeric subunit. Typically a native antibody (e.g., an IgG) has two such subunits, which are also held together by disulfide bonds. Within each chain, units of about 110 amino acid residues fold so as to form compact domains. Each domain is held together by a single intrachain disulfide bond. L chains have two domains, while H chains have four or five. Most H chains have a hinge region after the first (i.e., most amino-terminally located) two domains. The disulfide bonds linking together the heterodimeric subunits are located at the hinge regions. The hinge region is particularly sensitive to proteolytic cleavage, such proteolysis yielding two or three fragments (depending on the precise site of cleavage), a non-antigen binding fragment containing only H chain C regions (Fc) and one bivalent (Fab'2) or two monovalent (Fab) antigen binding fragments. The hinge region allows the antigen binding regions (each made up of a light chain and the first two domains of a heavy chain) to move freely relative to the rest of the native antibody, which includes the remaining heavy chain domains.

The first domain of each chain is highly variable in amino acid sequence, providing the vast spectrum of antibody binding specificities found in each individual. These are known as variable heavy (VH) and variable light (VL)

domains. The second and subsequent (if any) domains of each chain are relatively invariant in amino acid sequence. These are known as constant heavy (CH) and constant light (CL) domains.

Each variable region contains three loops of hypervariable sequence that provide a complementary structure to that of the antigen and are critical in determining the antigen binding specificity of the antibody, as they are the contact sites for binding to the antigen. These loops are known as complementarity determining regions, or CDRs. Each variable domain is made up of three CDRs embedded in four much less variable framework segments (FRs). Together, the sets of collinear CDRs and FRs are in large part responsible for determining the three dimensional conformation of the variable regions of antibody molecules.

CDRs and FRs are features that have been deduced from structural properties of antibody variable regions. Both amino acid sequence (primary structure) and three dimensional modeling (deduced secondary and tertiary structure) of antibody variable regions have been used by various researchers to define CDRs and, by default, FRs. While the positions of the CDRs are beyond question, not all workers in the art agree upon the precise locations of the boundaries of each CDR in VH or VL regions; there is no clear cut structural marker delineating CDR/FR boundaries.

Two definitions of CDR location are currently in general use in the art. These are the "sequence variability" definition of Kabat et al. ("Sequences of Proteins of Immunological Interest," 4th ed. Washington, D.C.: Public Health Service, N.I.H.) and the "structural variability" definition of Chothia and Lesk (J. Mol. Biol. 1987, 196:901). As used herein, the terms VL CDR1, VL CDR2, VL CDR3, VH CDR1, VH CDR2, and VH CDR3 refer minimally to the region of overlap between the regions designated for each CDR by each of these two definitions, and maximally to the total region spanned by the combination of the regions designated for each CDR by each of these two definitions.

One problem that antibody engineering attempts to address is the immune activity of a human patient that occurs in response to a native murine (or other non-human animal) antibody, typically a mAb, that is being administered to the patient for therapeutic purposes. This activity against murine antibodies is characterized by a human anti-mouse antibody (HAMA) response that can have deleterious effects on treatment efficacy and patient health. It has been found that almost all such human anti-non-human antibody ("HAMA type") activity is directed at the constant domains and at the FR regions of the variable domains of native non-human antibodies.

By manipulating the nucleic acid molecules encoding antibody H and L chains it is possible to incorporate non-human variable regions into antibodies otherwise made up of human constant regions. The resulting antibodies are referred to as "chimeric antibodies," and are typically less prone to eliciting HAMA type responses than are the non-human antibodies from which the variable regions are derived.

An even more effective approach to eliminating the potential of a non-human antibody to elicit a HAMA type response is to "humanize" it, i.e., to replace its non-human framework regions with human ones. One way of achieving such humanization involves the insertion of polynucleotide fragments encoding the non-human CDRs of the antibody to be humanized into a nucleic acid molecule encoding an otherwise human antibody (with human constant regions if desired) so as to replace the human CDRs and to use the resulting nucleic acid molecule to express the encoded "humanized" antibody.

Unfortunately, however, humanization of non-human antibodies has unpredictable effects on antibody antigen interactions, e.g., antigen binding properties. Some of this unpredictability stems from the properties of the CDRs. Certain CDRs may be more amenable to the construction of humanized antibodies that retain the properties of the non-human CDR donor antibody than others. While the CDRs are key to the antigen binding properties of an antibody, CDRs and FRs must interact appropriately if the antigen specificity of an antibody is to be retained following humanization. The effects of combination with particular human FRs on uncharacterized non-human CDRs cannot be reliably predicted by any known method. However, the successful humanization of an antibody provides information that, in general, facilitates the successful humanization of the CDRs of that antibody using other human or altered human FRs. In addition, approaches are available that facilitate tailoring human FRs to enhance the likelihood of successful humanization.

Other problems addressed by antibody engineering include efficient antibody production and alteration of antibody pharmacokinetics. Recombinant protein production is generally most efficiently carried out in bacterial hosts. The large size and multimeric nature of native antibodies makes their production in bacteria difficult. One approach to dealing with production problems is to use recombinant DNA methods to construct antibodies that have their H and L chains joined by a linker peptide to form a single chain (sc) antibody. As described below, there are several types of sc antibodies that can be constructed.

As is the case for humanization, the effects on antigen binding properties of constructing a particular type of sc antibody using H and L chains that have not been characterized with regard to their ability to function as part of an sc antibody cannot be reliably predicted by any known method. However, the successful construction of any one type of sc antibody from a particular native antibody provides information that, in general, facilitates the successful construction of other types of sc antibodies from that native antibody.

Single chain antibodies may include one each of only VH and VL domains, in which case they are referred to as scFv antibodies; they may include only one each of VH, VL, CH, and CL domains, in which case they are referred to as scFab antibodies; or they may contain all of the variable and constant regions of a native antibody, in which case they are referred to as full length sc antibodies.

The differing sizes of these antibodies imparts each with differing pharmacokinetic properties. In general, smaller proteins are cleared from the circulation more rapidly than larger proteins of the same general composition. Thus, full length sc antibodies and native antibodies generally have the longest duration of action, scFab antibodies have shorter durations of action, and scFv antibodies have even shorter durations of action. Of course, depending upon the illness being treated, longer or shorter acting therapeutic agents may be desired. For example, therapeutic agents for use in the prevention of immune and hemostatic disorders associated with extracorporeal circulation procedures (which are typically of brief duration) are preferably relatively short acting, while antibodies for the treatment of long term chronic conditions (such as inflammatory joint disease or GN) are preferably relatively long acting.

Detailed discussions of antibody engineering may be found in numerous recent publications including: Borrebaek, "Antibody Engineering, A Practical Guide," 1992, W.H. Freeman and Co. NY; and Borrebaek, "Antibody Engineering," 2nd ed. 1995, Oxford University Press, NY, Oxford.

SUMMARY OF THE INVENTION

In view of the foregoing, it is an object of the present invention to provide a new approach for reducing the glomerular inflammation and kidney dysfunction associated with GN.

The method of the invention involves the use of preparations containing antibodies to human complement component C5 as pharmaceutical agents. More particularly, the invention provides for the use of anti-C5 antibodies that bind to complement component C5 or active fragments thereof. Preferably, the antibodies block the generation and/or activity of complement components C5a and C5b. For most applications, the antibody is a monoclonal antibody.

In the preferred embodiments of the invention, the administration of the anti-C5 antibody preparation is started after the appearance of GN symptoms, e.g., after the appearance of proteinuria. Alternatively, the invention can be used prophylactically to treat patients who are at risk for an acute exacerbation of existing GN, e.g., patients experiencing a flare-up of symptoms of systemic lupus erythematosus or similar autoimmune diseases that have resulted in GN.

As shown in the examples presented below, anti-C5 antibodies administered subsequent to the onset of GN essentially eliminate glomerular inflammation/enlargement and reduce kidney dysfunction (see Examples 1 and 2).

Although not wishing to be bound by any particular theory of operation, it is believed that the anti-C5 antibodies have these and other therapeutic effects through their activity in blocking the generation or activity of the C5a and/or C5b active fragments of complement component C5. Through this blocking effect, the antibodies inhibit the proinflammatory (anaphylatoxic) effects of C5a and the generation of the C5b-9 membrane attack complex (MAC). Significantly, the blockage effected by the anti-C5 antibodies, since it occurs at the level of complement component C5, has the advantage of maintaining important opsonic, anti-infective, and immune complex clearance functions of the complement system mediated by, inter alia, complement component C3.

The invention additionally provides compositions comprising anti-C5 antibodies that block complement hemolytic activity and C5a generation. These antibodies are useful for the treatment of GN as well as a number of other conditions. These include treatment of immune and hemostatic dysfunctions associated with extracorporeal circulation (see copending U.S. patent application Ser. No. 08/217,391, now U.S. Pat. No. 5,853,722 which is incorporated herein by reference), treatment of inflammatory joint diseases (see copending U.S. patent application Ser. No. 08/311,489, which is incorporated herein by reference), and other complement associated conditions, particularly inflammatory diseases.

Although other antibodies can be used to treat GN in accordance with the present invention, the novel antibodies of the invention are preferred. Preferably, these novel antibodies bind to the alpha chain of C5, but do not exhibit substantial binding to the alpha chain cleavage product C5a (referred to hereinafter and in the claims as "free C5a"). Other preferred targets for antibody binding include fragments of the alpha chain of human C5 that are immunoreactive with the most preferred antibody of the invention, the 5G1.1 antibody discussed below. Such preferred targets include the 46 kDa acid hydrolysis fragment of C5 (the "5G46k" fragment), the 27 kDa tryptic digestion fragment of C5 (the "5G27k" fragment), the 325aa peptide spanning amino acid residues 725–1049 of SEQ ID NO:2 (the "5G325aa" peptide), the 200 amino acid peptide spanning amino acids residues 850 to 1049 of SEQ ID NO:2 (the "5G200aa" peptide)—as discussed below in Example 13.

The novel antibodies of the invention include antibodies that bind to an epitope within the amino acid sequence Val Ile Asp His Gln Gly Thr Lys Ser Ser Lys Cys Val Arg Gln Lys Val Glu Gly Ser Ser, (SEQ ID NO:1) hereinafter referred to as the KSSKC epitope. These novel antibodies that bind to the KSSKC epitope (SEQ ID NO:1) are hereinafter referred to as anti-KSSKC antibodies, and monoclonal antibodies binding to the KSSKC epitope are hereinafter referred to as anti-KSSKC mAbs.

The novel antibodies of the invention have many advantages over other anti-C5 antibodies, particularly with regard for their use as anti-inflammatory therapeutic agents. These include the ability to substantially block both complement hemolytic activity and the generation of the proinflammatory complement cleavage product C5a to substantially the same extent at the same concentration of antibody. Some of the preferred antibodies of the invention have the additional advantageous property of blocking the binding of C5 to C3 or C4.

Particularly preferred antibodies of the invention are monospecific native anti-KSSKC antibodies. The 5G1.1 native anti-KSSKC mAb has the distinct advantage of substantially blocking both complement hemolytic activity and the generation of C5a at a stoichiometric ratio of antibody to C5 that approaches the theoretical one to two (antibody to antigen) limit of binding that can be achieved by a bivalent antibody. This is a desirable property because it allows smaller doses of antibody to achieve therapeutic effects than would be required of otherwise similar antibodies that cannot function at such a ratio.

The invention further provides recombinant mAbs that are derivatives (including monovalent derivatives) of these native mAbs. These include anti-KSSKC recombinant mabs. Preferably the antibodies of the invention provide a level of blockade of both complement hemolytic activity and C5a generation (on a per mole of binding site basis) that is obtained when the antibody concentration is within an order of magnitude of that of the native mAbs. Particularly preferred anti-KSSKC recombinant mAbs provide a level of such blockade when the antibody concentration is no more than three fold that of the native mAbs of the invention.

The invention further provides nucleic acid sequences of polynucleotides encoding such recombinant anti-KSSKC mAbs, as well as amino acid sequences of the polypeptides encoded by these nucleic acid molecules of the invention.

The invention further provides CDR sequences that are useful in the construction of the humanized antibodies of the invention, as well as peptides and oligopeptides that are useful in the preparation and characterization of the antibodies of the invention.

Anti-C5 antibodies of the invention have activity in blocking the generation or activity of the C5a and/or C5b active fragments of complement component C5. Through this blocking effect, the antibodies inhibit the proinflammatory (anaphylatoxic) effects of C5a and the generation of the C5b-9 membrane attack complex (MAC). Significantly, the blockage effected by the anti-C5 antibodies, since it occurs at the level of complement component C5, has the advantage of maintaining important opsonic, anti-infective, and immune complex clearance functions of the complement system mediated by, inter alia, complement component C3.

The accompanying figures, which are incorporated in and constitute part of the specification, illustrate certain aspects

of the invention, and together with the description, serve to explain the principles of the invention. It is to be understood, of course, that both the figures and the description are explanatory only and are not restrictive of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A, 1B, and 1C—Photomicrographs of PAS stained sections of mouse kidneys. FIG. 1A—uninduced untreated mouse. FIG. 1B—GN-induced PBS-(control)-treated mouse. FIG. 1C—GN-induced anti-C5 treated mouse. Magnification for each is the same, approximately 400×.

FIGS. 2A, 2B, and 2C—Photomicrographs of immunofluorescence stained sections of mouse kidneys. FIG. 2A—uninduced untreated mouse. FIG. 2B—GN-induced PBS-(control)-treated mouse. FIG. 2C—GN-induced anti-C5 treated mouse. Magnification for each is the same, approximately 200×.

FIG. 3—Results of hemolytic (cell lysis) assays of serum from GN-induced animals treated with either anti-C5 antibodies in PBS ("Anti-C5") or PBS alone ("PBS control"). Also shown are the results of assays performed with normal serum.

FIG. 4—Results of soluble C5b-9 ("sC5b-9") assays. "ND" indicates not determined.

FIGS. 5A, 5B, and 5C—Immunofluorescence photomicrographs of kidney sections stained for mouse C3. FIG. 5A—uninduced untreated mouse. FIG. 5B—GN-induced PBS-(control)-treated mouse. FIG. 5C—GN-induced anti-C5 treated mouse. Magnification for each is the same, approximately 400×.

FIG. 6—Results of C3a assays of samples of circulating human blood. "ND" indicates not determined.

FIGS. 7A and 7B—Pharmacokinetic analyses of the reduction of the cell lysis ability of mouse (FIG. 7A) or human (FIG. 7B) blood after treatment with anti-C5 antibodies.

The immunofluorescent staining of FIGS. 2 and 5 is confined to the glomerular capillary network (tuft) and thus the enlargement of the glomerulus seen in FIG. 1B is not visible in FIGS. 2B and 5B.

FIG. 8—Scatchard analysis of native 5G1.1 binding to C5.

FIG. 9—Scatchard analysis of native N19/8 binding to C5.

FIG. 10—C3a generation in samples of circulating human blood in the presence of native 5G1.1.

FIG. 11—sC5b-9 generation in samples of circulating human blood in the presence of native 5G1.1.

FIG. 12—Serum hemolytic activity of samples of circulating human blood in the presence of native 5G1.1.

FIG. 13—Serum hemolytic activity in the presence of m5G1.1 scFv.

FIG. 14—C5a generation in the presence of m5G1.1 scFv.

FIG. 15—C3a generation in samples of circulating human blood in the presence of m5G1.1 scFv.

FIG. 16—Serum hemolytic activity of samples of circulating human blood in the presence of 5G1.1 scFv.

FIG. 17—sC5b-9 generation in samples of circulating human blood in the presence of m5G1.1 scFv.

FIG. 18—The light chain variable region of the antibody 5G1.1. Sequence derived from the 5' oligonucleotide primer used for PCR amplification of the variable region is shown in lower case. Amino acids are number according to Kabat et al., supra. Boxed amino acids correspond to peptide sequences obtained from the mature 5G1.1 light chain or from an endoproteinase Lys C peptide of 5G1.1. The complementarity determining region (CDR) residues according to the sequence variability definition and the structural variability definition are underlined and overlined, respectively.

FIG. 19—The heavy chain variable region of the antibody 5G1.1. Sequence derived from the 5' oligonucleotide primer used for PCR amplification of the variable region is shown in lower case. Amino acids are numbered using the scheme of Kabat et al. supra with +1 denoting the first amino acid of the processed mature variable region. Boxed amino acids correspond to peptide sequence obtained from the 5G1.1 heavy chain after treatment with pyroglutamate aminopeptidase. The complementarity determining region (CDR) residues according to the sequence variability definition or according to the structural variability definition are underlined and overlined, respectively.

BACKGROUND PHYSIOLOGY & PATHOLOGY

The discussion in this section is not limited to subject matter that qualifies as "prior art" against the present invention. Therefore, no admission of such prior art status shall be implied or inferred by reason of inclusion of particular subject matter in this discussion, and no declaration against the present inventors' interests shall be implied by reason of such inclusion.

I. Introduction

As described above, the present invention relates to therapeutic treatments for GN and other immune complex mediated diseases, as well as to the treatment of other complement mediated diseases and to the inhibition of complement component C5. To provide background for the description of the preferred embodiments and the examples presented below, we turn first to general discussions of the complement arm of the immune system, the pathophysiologic features of GN, and previous studies of the role of complement in GN pathogenesis.

General discussions of the complement system and GN can be found in, for example, Glassock and Brenner, 1994; Couser, 1993; Couser, 1992; Couser, et al, 1992; Rich, 1992; Glassock and Brenner, 1987; Robbins and Cotran, 1979; and Guyton, 1971.

II. The Complement System

The complement system acts in conjunction with other immunological systems of the body to defend against intrusion of cellular and viral pathogens. There are at least 25 complement proteins, which are found as a complex collection of plasma proteins and membrane cofactors. The plasma proteins make up about 10% of the globulins in vertebrate serum. Complement components achieve their immune defensive functions by interacting in a series of intricate but precise enzymatic cleavage and membrane binding events. The resulting complement cascade leads to the production of products with opsonic, immunoregulatory, and lytic functions.

The complement cascade progresses via the classical pathway or the alternative pathway. These pathways share many components, and while they differ in their initial steps, they converge and share the same "terminal complement" components (C5 through C9) responsible for the activation and destruction of target cells.

The classical complement pathway is typically initiated by antibody recognition of and binding to an antigenic site on a target cell. The alternative pathway is usually antibody

independent, and can be initiated by certain molecules on pathogen surfaces. Both pathways converge at the point where complement component C3 is cleaved by an active protease (which is different in each pathway) to yield C3a and C3b. Other pathways activating complement attack can act later in the sequence of events leading to various aspects of complement function.

C3a is an anaphylatoxin (see discussion below). C3b binds to bacterial and other cells, as well as to certain viruses and immune complexes, and tags them for removal from the circulation. (C3b in this role is known as opsonin.) The opsonic function of C3b is considered to be the most important anti-infective action of the complement system. Patients with genetic lesions that block C3b function are prone to infection by a broad variety of pathogenic organisms, while patients with lesions later in the complement cascade sequence, i.e., patients with lesions that block C5 functions, are found to be more prone only to Neisseria infection, and then only somewhat more prone (Fearon, in *Intensive Review of Internal Medicine,* 2nd Ed. Fanta and Minaker, eds. Brigham and Women's and Beth Israel Hospitals, 1983).

C3b also forms a complex with other components unique to each pathway to form classical or alternative C5 convertase, which cleaves C5 into C5a and C5b. C3 is thus regarded as the central protein in the complement reaction sequence since it is essential to both the alternative and classical pathways (Wurzner, et al., *Complement Inflamm.* 8:328–340, 1991). This property of C3b is regulated by the serum protease Factor I, which acts on C3b to produce iC3b. While still functional as opsonin, iC3b cannot form an active C5 convertase.

C5 is a 190 kDa beta globulin found in normal serum at approximately 75 μg/ml (0.4 μM). C5 is glycosylated, with about 1.5–3 percent of its mass attributed to carbohydrate. Mature C5 is a heterodimer of a 999 amino acid 115 kDa alpha chain that is disulfide linked to a 656 amino acid 75 kDa beta chain. C5 is synthesized as a single chain precursor protein product of a single copy gene (Haviland et al. J. Immunol. 1991, 146:362–368). The cDNA sequence of the transcript of this gene predicts a secreted pro-C5 precursor of 1659 amino acids along with an 18 amino acid leader sequence (SEQ ID NO:2).

The pro-C5 precursor is cleaved after amino acid 655 and 659, to yield the beta chain as an amino terminal fragment (amino acid residues +1 to 655 of SEQ ID NO:2) and the alpha chain as a carboxyl terminal fragment (amino acid residues 660 to 1658 of SEQ ID NO:2), with four amino acids (amino acid residues 656–659 of SEQ ID NO:2) deleted between the two.

C5a is cleaved from the alpha chain of C5 by either alternative or classical C5 convertase as an amino terminal fragment comprising the first 74 amino acids of the alpha chain (i.e., amino acid residues 660–733 of SEQ ID NO:2). Approximately 20 percent of the 11 kDa mass of C5a is attributed to carbohydrate. The cleavage site for convertase action is at or immediately adjacent to amino acid residue 733 of SEQ ID NO:2. A compound that would bind at or adjacent to this cleavage site would have the potential to block access of the C5 convertase enzymes to the cleavage site and thereby act as a complement inhibitor.

C5 can also be activated by means other than C5 convertase activity. Limited trypsin digestion (Minta and Man, J. Immunol. 1977, 119:1597–1602; Wetsel and Kolb, J. Immunol. 1982, 128:2209–2216) and acid treatment (Yammamoto and Gewurz, J. Immunol. 1978, 120:2008; Damerau et al., Molec. Immunol. 1989, 26:1133–1142) can also cleave C5 and produce active C5b.

C5a is another anaphylatoxin (see discussion below). C5b combines with C6, C7, and C8 to form the C5b-8 complex at the surface of the target cell. Upon binding of several C9 molecules, the membrane attack complex (MAC, C5b-9, terminal complement complex—TCC) is formed. When sufficient numbers of MACs insert into target cell membranes the openings they create (MAC pores) mediate rapid osmotic lysis of the target cells. Lower, non-lytic concentrations of MACs can produce other effects. In particular, membrane insertion of small numbers of the C5b-9 complexes into endothelial cells and platelets can cause deleterious cell activation. In some cases activation may precede cell lysis.

As mentioned above, C3a and C5a are anaphylatoxins. These activated complement components can trigger mast cell degranulation, which releases histamine and other mediators of inflammation, resulting in smooth muscle contraction, increased vascular permeability, leukocyte activation, and other inflammatory phenomena including cellular proliferation resulting in hypercellularity. C5a also functions as a chemotactic peptide that serves to attract pro-inflammatory granulocytes to the site of complement activation.

III. Pathophysiology of GN

Although GN may accompany an extraordinary range of pathologic processes, in general it is encountered most commonly in the course of infectious diseases, in autoimmunity, and as a consequence of therapy for some other disease process. The causative mechanism for GN is typically the deposit of circulating immune complexes in the kidney. Factors involved in the pathogenesis of GN include the specific antigen and antibody involved and the inflammatory processes that occur as a consequence of immune complex deposition.

Antigens Involved in the Formation of Immune Complexes that Cause GN

Antigens involved in the development of GN can be broadly classified as endogenous, infectious, and iatrogenic (those encountered as a consequence of medical practice). In many cases the specific antigen is unknown, although the general class can usually be identified.

The best known example of the formation of endogenous immune complexes is the DNA anti-DNA complexes produced in connection with systemic lupus erythematosus (lupus, SLE). Other important sources of endogenous antigens include malignancies in which immune complex formation may contribute to the development of paraneoplastic syndromes.

Infections with organisms of many types, particularly chronic infections, are also associated with the development of immune complexes that can cause GN. Bacterial and fungal infections that can produce such complexes include infection with certain strains of streptococci, Pseudomonas, disseminated gonococcal infection, lepromatous leprosy, subacute bacterial endocarditis, bronchopulmonary aspergillosis, secondary syphilis, and chronic infections in patients with cystic fibrosis.

Viral diseases in which immune complex deposition may be a prominent feature include hepatitis B infection, dengue, infectious mononucleosis, and subacute sclerosing panencephalitis. GN is also a prominent feature of many parasitic infestations such as the GN seen in children with quartan malaria, as well as toxoplasmosis, trypanosomiasis, and schistosomiasis.

Iatrogenic antigens constitute a special class of exogenous antigens. These include those responsible for the prototype immune complex disease, serum sickness, which follows

formation of immune complexes between heterologous serum constituents and autologous antibodies. Serum sickness was regularly seen earlier in this century when infectious diseases were frequently treated with heterologous antisera.

An iatrogenic disease essentially indistinguishable from classic serum sickness can occur as a consequence of high-dose antibiotic therapy. The serum sickness-like manifestations of immune responses to these drugs include GN and reflect the fact that certain drugs, particularly the β-lactam and sulfonamide antibiotics, are effective haptens that are capable of inducing antibody responses upon spontaneous conjugation to autologous proteins.

Factors Affecting Immune Complex Formation and Deposition

Features of both antigen and antibody determine the likelihood of pathologic immune complex formation and subsequent deposition in the kidney. Chief among these are the absolute concentrations of the reactants and their relative molar ratios.

Most antigens display multiple epitopes and typically stimulate a polyclonal antibody response. All naturally occurring antibody molecules are at least bivalent. These properties allow for the formation of an extensive antigen-antibody lattice, the size of which is determined largely by the affinity of the antibodies and the molar ratio of antigen to antibody.

In general, antibody responses begin under conditions in which antigen is present in excess to antibody, and this relative ratio changes as the antibody response increases in magnitude. Complexes formed initially are usually small and exhibit little or no pathogenic activity. In contrast, very large complexes are often formed as the amount of antigen becomes limiting, late in the course of an antibody response under conditions of antibody excess. Because these very large complexes are readily cleared by the reticuloendothelial system in the liver, they are also relatively nonpathogenic.

The formation of immune complexes that can cause GN is believed to occur during conditions of slight antigen excess or near the point of antibody-antigen equivalence, where lattice formation is maximal and lattice size is large, but not very large.

Several factors influence the speed and location of immune complex precipitation. Interactions between Fc regions of antibody molecules promote rapid precipitation of immune complexes. The role of Fc-Fc interactions in immune complex precipitation is illustrated by studies of the properties of F(ab')2 antibody fragments, which do not contain Fc regions. Although the valence of F(ab')2 fragments does not differ from that of most whole immunoglobulins, F(ab')2 antibody fragments form lattices more slowly.

Antigen charge plays a role in determining the tissue localization of sites of deposition of immune complex precipitates. Complexes with a substantial positive charge are preferentially attracted to the strong negative charge of basement membranes, particularly in the renal glomerulus.

Localized presence of antigen may largely account for certain cases of organ specific immune complex deposition. Diseases such as Goodpasture's syndrome (a rare form of GN) are typically not classified as immune complex diseases because the complexes are formed in situ in the kidney rather than being preformed in the circulation and then deposited. Once the immune complexes are formed, the subsequent inflammatory process is believed to be essentially the same as that seen following deposition of preformed complexes. However, the different mode of deposition distinguishes this syndrome from typical GN caused by circulating immune complexes.

Features of blood flow and vascular structure are also important in determining the localization of immune complex deposits. Chief among these is capillary permeability. Because their capillary endothelium is fenestrated, renal glomeruli are preferential sites for the deposition of immune complexes. Hemodynamic variables enhancing immune complex localization include turbulence of flow and increased blood pressure, both of which are present in the renal glomeruli.

Complement and Complement Receptors as Reaulators of Immune Complex Deposition

In addition to their proinflammatory functions, complement components can also inhibit immune complex deposition and resolubilize immune complex precipitates from sites of deposition. In addition, it is known that erythrocyte receptors for C3b, e.g., CR1, are important for reticuloendothelial clearance of opsonized circulating immune complexes.

Analysis of the clinical pattern of immune complex disease in patients with deficiencies of particular complement components provides information regarding the normal role of these components in the prevention of complex deposition. The incidence of immune complex disease in patients with deficiencies of Clq, Clr, Cls, C4, C2, or C3 varies from 60 to 90 percent, with the majority of these patients exhibiting a lupus-like syndrome. Immune complex disease is rarely associated with deficiencies of late-acting or alternative pathway components.

The binding of complement components to immune complexes prevents the formation of large antigen-antibody lattices and inhibits immune precipitation. This process requires activation via the classical pathway; serum that is deficient for Clq, C4, or C2 does not effectively inhibit lattice formation and complex precipitation. Classical pathway dependence may reflect the initial binding of Cl components, impeding the Fc-Fc interactions between IgG molecules that contribute to immune precipitation. This is followed by covalent binding of C3b to the complexes, which further inhibits immune precipitation and leads to solubilization of previously deposited complexes.

The solubilization process also depends upon activation of components of the alternative pathway. Consequently, by promoting clearance of immune complexes and inhibiting their deposition at sites of inflammation, complement components and their receptors serve as negative regulators of immune complex diseases that may retard disease development.

It should be noted that the present invention involves blocking the activities of complement component C5. The targeting of this component does not alter the functions of the early complement components, and thus does not compromise the negative regulatory effects on immune complex deposition of those early components.

Immune Complex-Mediated Inflammation

Basophils are important in the initiation of immune complex-mediated inflammatory responses, as capillary permeability is markedly increased by the action of vasoactive amines such as histamine and platelet-activating factor, which are released by these cells. Vascular permeability is also promoted by aggregation of platelets at sites of an inflammatory lesion, with the release of platelet-activating factor and the formation of microthrombi.

Basophil degranulation may reflect the effects of IgE antibodies, as well as the elaboration of the anaphylatoxin components of complement, C3a and C5a.

In addition to basophils and platelets, the primary cellular effectors of immune complex-mediated inflammation are polymorphonuclear leukocytes, monocytes, and macrophages.

IV. Previous Studies of the Role of Complement in GN Pathoaenesis

Extensive work has been performed in an attempt to understand the possible role of complement in the development of GN. This work has included studies of GN using a number of animal models by, among others, Unanue, et al., (1964); Cochrane, et al., (1965); Kniker, et al., (1965); Salant, et al., (1980); Groggel, et al., (1983); Falk and Jennette (1986); Jennette, et al., (1987); Passwell, et al., (1988); Schrijver, et al., (1988); Baker, et al., (1989); Schrijver, et al., (1990); Couser, et al., (1991); and Couser, et al., (1992).

These studies have shown that complement plays a role in GN pathogenesis. However, they have not established specific unequivocal roles for the various complement components. In particular, the relative roles of C3 and other anaphylatoxins compared to the roles of the terminal complement components in GN pathogenesis have not been unequivocally established. Also, some researchers have reported that complement depletion does not diminish glomerular injury. See Kniker, et al., (1965).

The foregoing work includes that of Falk and Jennette (1986), who reported results of experiments in which attempts were made to induce GN in mice having a genetic defect that resulted in a deficiency of complement component C5. The report concludes that C5 or some terminal complement component dependent on C5 plays a role in the pathogenesis of GN.

Significantly, with regard to the present invention, Falk and Jennette in no way disclose or suggest that an antibody to C5 can be used to treat GN. Indeed, it would be counterintuitive to use an antibody to treat disease which typically involves the formation and deposition of circulating antibody-antigen immune complexes. Plainly, the creation of more circulating immune complexes would seem to be the last way to go to solve a problem that can be caused by circulating immune complexes. Yet, as demonstrated by the surprising results presented below, anti-C5 antibodies have been found to effectively block GN, even though the creation of additional circulating immune complexes is inherent in their mode of action.

Baker et al. (1989), Couser et al. (1991), and Couser et al. (1992) (hereinafter referred to collectively as the "C6" work) discuss experiments in which high levels of an anti-C6 polyclonal antibody preparation were administered to rats, following which immune complexes were formed in situ in the rats' kidneys. Significantly, with regard to the present invention, the anti-C6 antibody preparation was not administered to animals with pre-existing kidney disease, i.e., it was not used as a therapeutic treatment. Moreover, the experimental protocol used in the C6 experiments did not involve circulating immune complexes, but rather involved complexes formed in situ. Accordingly, the experiments did not disclose or suggest the counterintuitive approach of the present invention wherein more circulating immune complexes are formed in the process of treating a disease state caused by circulating immune complexes.

Further, the anti-C6 antibody dosages used in the C6 work were too high for practical medical use. Specifically, these antibodies were used at a dosage of 1 gm/kg, a dosage which would correspond to 70 gm of antibody for a 70 kg (155 lb) individual. In contrast, the anti-C5 antibodies used in the practice of the present invention are used at concentrations at or below 0.1 gm/kg, i.e., a factor of at least ten times less than used in the C6 work. Indeed, as shown by the examples presented below, anti-C5 antibody dosages as low as 0.03 gm/kg, i.e., 33 times less than those used in the C6 work, have been found to achieve the therapeutic effects of the invention in treating GN. For a 70 kg individual, this antibody level corresponds to a dose of just 2.1 gms.

The novel anti-KSSKC antibodies of the invention allow the use of even lower dosage levels to treat GN and other inflammatory conditions. Based upon their level of activity in human blood, they are expected to provide complete complement inhibition at dosages below 0.005 g/kg, and to provide therapeutically effective complement inhibition at dosages below 0.003 g/kg. This 3 mg/kg dosage is one tenth the dosage discussed below in Examples 4 and 5 for the for the anti-C5 (beta chain specific) mAb N19/8. Some of the full length anti-KSSKC mabs of the invention will provide therapeutic benefits even at dosages below 0.0022 g/kg. This is the minimum dose providing complete complement inhibition as calculated from the data obtained using the anti-KSSKC 5G1.1 mAb in human blood in a CPB circuit, as discussed below in Example 9.

Accordingly, dosages of less than 0.005 g/kg are preferred, with dosages of below 0.003 g/kg being more preferred, and dosages below 0.0022 g/kg being particularly preferred. For a 70 kg individual, these antibody dosage levels correspond to a dose of less than 0.35 gms for the highest dosage of the preferred dosages, less than 0.21 gms for the more preferred dosage, and less than or equal to 0.15 gms for the most preferred dosage.

Of course, dosage levels of single chain and other recombinant mAbs of the invention must be adjusted according to their level of activity (e.g., their binding affinity, their ability to block C5 activation, and/or their ability to block complement hemolytic activity), their valency, and their molecular weight. For example, the humanized scFv anti-KSSKC mAbs of Example 11 are approximately 27 kDa, about one sixth the approximately 155 kDa mass of a native, full length IgG antibody. These antibodies completely block complement hemolytic activity and C5a generation at a ratio of 3:1, six fold greater than for native 5G1.1 (but only three fold greater when viewed in terms of numbers of antibody-antigen binding sites).

Thus, the number of molecules of each of these scFvs required to equal the effect of a single molecule of native 5G1.1 must be increased by a factor of six to adjust for the ratio at which blocking is complete. Since the mass of these molecules is approximately one sixth of the mass of native 5G1.1, dosages of the scFvs are in the same range as those for the native 5G1.1 mAb.

In addition to lowering dosage levels, the anti-C5 antibodies used in the practice of the present invention (i.e., in treating GN) achieve important therapeutic effects not achieved with the anti-C6 antibodies. Specifically, the control and test animals in the C6 work exhibited both hypercellularity and narrowing of capillary lumens. In direct contrast, no such hypercellularity or narrowing of capillary lumens was seen when diseased individuals were treated with anti-C5 antibodies (see FIG. 1).

Moreover, the anti-C5 antibodies used in the present invention achieve a reduction in glomerular enlargement, thus providing a clear demonstration of the unexpectedly powerful anti-inflammatory effects of the anti-C5 antibodies used in the practice of the invention. Nowhere in the C6 work is there any disclosure or suggestion of such a powerful anti-inflammatory effect.

V. Anti-C5 Monoclonal Antibodies that Block Complement Hemolytic Activity and Block the Generation of C5a

Anti-C5 mabs that have the desirable ability to block complement hemolytic activity and to block the generation of C5a (and are thus preferred for use in the treatment of GN and other inflammatory conditions in accordance with the present invention) have been known in the art since at least 1982 (Moongkarndi et al. Immunobiol. 1982, 162:397; Moongkarndi et al. Immunobiol. 1983, 165:323). Antibodies known in the art that are immunoreactive against C5 or C5 fragments include antibodies against the C5 beta chain (Moongkarndi et al. Immunobiol. 1982, 162:397; Moongkarndi et al. Immunobiol. 1983, 165:323; Wurzner et al. 1991, supra; Mollnes et al. Scand. J. Immunol. 1988, 28:307–312); C5a (see for example, Ames et al. J. Immunol. 1994, 152:4572–4581, U.S. Pat. No. 4,686,100, and European patent publication No. 0 411 306); and antibodies against non-human C5 (see for example, Giclas et al. J. Immunol. Meth. 1987, 105:201–209). Significantly, none of these anti-C5 mAbs has the properties of the novel anti-C5 mabs of the invention, i.e., none of them binds to the C5 alpha chain but not to the C5 cleavage product C5a, none of them has the ability to substantially block both complement hemolytic activity and the generation of C5a to substantially the same extent at the same concentration of antibody. It is noteworthy that an scFv derivative of the N19/8 antibody of Wurzner et al. 1991, supra, has been prepared, and that the N19/8 scFv has 50% less inhibitory activity towards C5a generation than the native N19/8 antibody (see Example 15). This is in contrast to the 5G1.1 scFv, which retained substantially all of its inhibitory activity towards C5a generation (see Example 12).

While not wishing to be bound by any particular theory of operation, it is believed that these distinctions are due to the specific binding characteristics of the antibodies of the invention. Accordingly, antibodies that do not bind to sites within the alpha chain of C5, and antibodies that bind to the C5 cleavage product C5a (free C5a), are believed to lack the ability to substantially block both complement hemolytic activity and the generation of C5a to substantially the same extent at the same concentration of antibody.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

As discussed above, the present invention relates to the use of anti-C5 antibodies in treating patients suffering from GN and other diseases, and to specific C5 antibodies and antibody preparations. Preferably, and when used to treat GN, the anti-C5 antibodies are used in an amount effective to substantially reduce (e.g., reduce by at least about 50%) the cell-lysing ability of complement present in the patient's blood (the "cell-lysing ability of complement present in the patient's blood" is also referred to herein as the "serum complement activity of the patient's blood"). Reduction of the cell-lysing ability of complement present in the patient's blood can be measured by methods well known in the art such as, for example, by the chicken erythrocyte hemolysis method described below under the heading "Cell Lysis Assays."

To achieve the desired reductions, the anti-C5 antibodies can be administered in a variety of unit dosage forms. The dose will vary according to the particular antibody. For example, different antibodies may have different masses and/or affinities, and thus require different dosage levels. Antibodies prepared as Fab' fragments will also require differing dosages than the equivalent intact immunoglobulins, as they are of considerably smaller mass than intact immunoglobulins, and thus require lower dosages to reach the same molar levels in the patient's blood.

The dose will also vary depending on the manner of administration, the particular symptoms of the patient being treated, the overall health, condition, size, and age of the patient, and the judgment of the prescribing physician. Dosage levels of the antibodies for human subjects are generally between about 1 mg per kg and about 100 mg per kg per patient per treatment, and preferably between about 5 mg per kg and about 50 mg per kg per patient per treatment. In terms of plasma concentrations, the antibody concentrations are preferably in the range from about 25 μg/ml to about 500 μg/ml.

Subject to the judgement of the physician, a typical therapeutic treatment includes a series of doses, which will usually be administered concurrently with the monitoring of clinical endpoints such as BUN levels, proteinuria levels, etc., with the dosage levels adjusted as needed to achieve the desired clinical outcome. Alternatively, levels of serum complement activity available in the patient's blood are monitored using the techniques set forth below under the heading "Cell Lysis Assays" to determine if additional doses or higher or lower dosage levels of antibodies are needed, with such doses being administered as required to maintain at least about a 50% reduction, and preferably about a 95% or greater reduction of serum complement activity. Other protocols can, of course, be used if desired as determined by the physician.

Administration of the anti-C5 antibodies will generally be performed by an intravascular route, e.g., via intravenous infusion by injection. Other routes of administration may be used if desired. Formulations suitable for injection are found in *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Philadelphia, Pa., 17th ed. (1985). Such formulations must be sterile and non-pyrogenic, and generally will include a pharmaceutically effective carrier, such as saline, buffered (e.g., phosphate buffered) saline, Hank's solution, Ringer's solution, dextrose/saline, glucose solutions, and the like. The formulations may contain pharmaceutically acceptable auxiliary substances as required, such as, tonicity adjusting agents, wetting agents, bactericidal agents, preservatives, stabilizers, and the like.

The formulations of the invention can be distributed as articles of manufacture comprising packaging material and the anti-C5 antibodies. When prepared for use in the treatment of GN, the packaging material will include a label which indicates that the formulation is for use in the treatment of kidney disease and may specifically refer to nephritis or glomerulonephritis.

The anti-C5 antibody is preferably a monoclonal antibody, although polyclonal antibodies produced and screened by conventional techniques can also be used if desired. As discussed above, the anti-C5 antibodies must be effective in reducing the cell-lysing ability of complement present in human blood. As also discussed above, this property of the antibodies can be determined by methods well known in the art such as, for example, by the chicken erythrocyte hemolysis method described below under the heading "Cell Lysis Assays".

The anti-C5 antibodies used in the practice of the invention bind to C5 or fragments thereof, e.g., C5a or C5b. Preferably, the anti-C5 antibodies are immunoreactive against epitopes on the beta chain of purified human complement component C5 and are capable of blocking the conversion of C5 into C5a and C5b by C5 convertase. This capability can be measured using the techniques described in

Wurzner, et al., *Complement Inflamm* 8:328–340, 1991. Preferably, the anti-C5 antibodies are used to treat GN in an amount effective to reduce the C5 convertase activity available in the patient's blood by at least about 50%.

In a particularly preferred embodiment of the invention, the anti-C5 antibodies are not immunoreactive against epitopes on the beta chain, but rather are immunoreactive against epitopes within the alpha chain of purified human complement component C5. In this embodiment the antibodies are also capable of blocking the conversion of C5 into C5a and C5b by C5 convertase. In an especially preferred example of this embodiment they can provide this blockade at substantially the same concentrations needed to block hemolytic activity.

Within the alpha chain, the most preferred antibodies bind to an amino-terminal region, however, they do not bind to free C5a. Particularly preferred targets for these antibodies within the alpha chain include the 5G46k fragment, the 5G27k fragment, the 5G325aa peptide, the 5G200aa peptide, or the KSSKC epitope. The scope of the invention also includes the 5G46k fragment, the 5G27k fragment, the 5G325aa peptide, the 5G200aa peptide, or the KSSKC epitope (SEQ ID NO:1) that are useful as immunogens and screening ligands for producing the antibodies of the invention.

Hybridomas producing monoclonal antibodies reactive with complement component C5 can be obtained according to the teachings of Sims, et al., U.S. Pat. No. 5,135,916. As discussed therein, antibodies are prepared using purified components of the complement membrane attack complex as immunogens. In accordance with the present invention, complement component C5 or C5b is preferably used as the immunogen. In accordance with a particularly preferred aspect of the present invention, the immunogen is the alpha chain of C5. Within the alpha chain, the most preferred immunogens include the 5G46k fragment, the 5G27k fragment, the 5G325aa peptide, or the 5G200aa peptide. A less preferred immunogen is the KSSKC epitope.

In accordance with the invention, the antibodies of the invention all share certain required functional properties. These are the ability to substantially inhibit complement hemolytic activity and to substantially inhibit the conversion of C5 to produce C5a. Preferably, but not requisitely, they provide these functions when used at a molar ratio of antibody to antigen (C5) of 3:1 or less.

A particularly preferred antibody of the invention is the 5G1.1 antibody (5G1.1, produced by the 5G1.1 hybridoma, ATCC designation HB-11625). Other particularly preferred antibodies of the present invention share the required functional properties discussed in the preceding paragraph and have any of the following characteristics:

(1) they compete with 5G1.1 for binding to portions of C5—the C5 alpha chain, the 5G46k fragment, the 5G27k fragment, the 5G325aa peptide (SEQ ID NO:1), the 5G200aa" peptide, or the KSSKC peptide—that are specifically immunoreactive with 5G1.1; and

(2) they specifically bind to the C5 alpha chain, the 5G46k fragment, the 5G27k fragment, the 5G325aa peptide, the 5G200aa" peptide, and/or the KSSKC peptide (SEQ ID NO:1). Such specific binding, and competition for binding can be determined by various methods well known in the art, including the plasmon surface resonance method (Johne et al., J. Immunol. Meth. 1993, 160:191–198).

(3) they block the binding of C5 to either C3 or C4 (which are components of C5 convertase).

Also in accordance with the invention, the antibodies preferably should prevent the cleavage of C5 to form C5a and C5b, thus preventing the generation of the anaphylatoxic activity associated with C5a and preventing the assembly of the membrane attack complex associated with C5b. In a particularly preferred embodiment, these anti-C5 antibodies will not impair the opsonization function associated with the activation of complement component C3 by a C3 convertase. Plasma C3 convertase activity can be measured by assaying plasma for the presence of C3a as described below under the heading "Histology." Preferably, the anti-C5 antibody produces essentially no reduction in plasma C3a levels.

General methods for the immunization of animals (in this case with C5 or C5b or another preferred immunogen), isolation of polyclonal antibodies or antibody producing cells, fusion of such cells with immortal cells (e.g., myeloma cells) to generate Hybridomas secreting monoclonal antibodies, screening of hybridoma supernatants for reactivity of secreted monoclonal antibodies with a desired antigen (in this case C5 or C5b or another preferred immunogen), the preparation of quantities of such antibodies in hybridoma supernatants or ascites fluids, and for the purification and storage of such monoclonal antibodies, can be found in numerous publications. These include: Coligan, et al., eds. *Current Protocols In Immunology*, John Wiley & Sons, New York, 1992; Harlow and Lane, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 1988; Liddell and Cryer, *A Practical Guide To Monoclonal Antibodies*, John Wiley & Sons, Chichester, West Sussex, England, 1991; Montz, et al., *Cellular Immunol.* 127:337–351, 1990; Wurzner, et al., *Complement Inflamm.* 8:328–340, 1991; and Mollnes, et al., *Scand. J. Immunol.* 28:307–312, 1988.

As used herein, the term "antibodies" refers to immunoglobulins produced in vivo, as well as those produced in vitro by a hybridoma, and antigen binding fragments (e.g., Fab' preparations) of such immunoglobulins, as well as to recombinantly expressed antigen binding proteins, including immunoglobulins, chimeric immunoglobulins, "humanized" immunoglobulins, antigen binding fragments of such immunoglobulins, single chain antibodies, and other recombinant proteins containing antigen binding domains derived from immunoglobulins. As used herein, "antibodies" also refers to antigen binding synthetic peptides comprising sequences derived from the sequences of immunoglobulin antigen binding domains. As used herein, the term "recombinant mAbs" refers to recombinantly expressed antigen binding proteins. As used herein, the term "antibody-antigen binding site" refers to an antigen binding site of an antibody comprising at least one CDR sequence.

Antibodies whose amino acid sequences are full length immunoglobulin sequences that have not been truncated (e.g., to produce an scFv or an Fab) or mutated (e.g., spliced to form a chimeric antibody or humanized) are referred to herein as "native" antibodies. Publications describing methods for the preparation of such antibodies, in addition to those listed immediately above, include: Reichmann, et al., *Nature,* 332:323–327, 1988; Winter and Milstein, *Nature,* 349:293–299, 1991; Clackson, et al., *Nature,* 352:624–628, 1991; Morrison, *Annu Rev Immunol,* 10:239–265, 1992; Haber, *Immunol Rev,* 130:189–212, 1992; and Rodrigues, et al., *J Immunol,* 151:6954–6961, 1993.

While treatment of GN in accordance with the process of the present invention may be carried out using polyclonal or monoclonal antibodies, monospecific antibodies are preferred. As used herein "monospecific antibodies" refer to

antibodies that bind to a specific region of a particular antigen. All monoclonal antibodies are monospecific, but polyclonal antibodies are typically not monospecific.

As is known in the art, however, monospecific polyclonal antibodies may be prepared by various methods. For example, a peptide (e.g., an oligopeptide—as used hereinafter and in the claims, a polymer of 5 to 200 amino acids) may be used as an immunogen. Another procedure allowing the preparation of monospecific polyclonal antibodies is the use of antigen affinity purification techniques to isolate a monospecific antibody population from a polyclonal antibody mixture. In accordance with the present invention, peptides are preferred as immunogens for the production and as affinity ligands for the purification of monospecific polyclonal anti-KSSKC antibodies.

The native (i.e., non-engineered) monoclonal antibodies of the invention are preferably prepared by conventional means, with the 5G46k fragment, the 5G27k fragment, the 5G200aa peptide, the 5G325aa peptide, and/or the KSSKC peptide (SEQ ID NO:1) (e.g., immobilized on a polypropylene membrane as described below in Example 13) being used as screening ligand(s). This involves testing hybridoma supernatants for binding to each screening ligand.

In one preferred embodiment, the native mAbs of the invention are prepared using the alpha chain of human C5, or fragments thereof, as immunogen. Preferred fragments of the alpha chain of human C5 for this purpose include the 5G46k fragment, the 5G27k fragment, and/or the 5G200aa fragment. Although less preferred, the KSSKC peptide (SEQ ID NO:1) may also be used as an immunogen.

Another (albeit less preferred) immunogen and screening ligand for the preparation of antibodies within the scope of the novel antibodies of the present invention is the "cleavage site peptide," i.e., the peptide spanning amino acids 725 through 754 of SEQ ID NO:2 (the C5a cleavage site), as discussed below in Example 13.

In another preferred embodiment of the invention, the native mAbs of the invention are prepared in transgenic mice expressing human immunoglobulins (see, for example, Green et al., Nature Genet. 1994, 7:13–21). In this case, the same preferred immunogens and screening ligands are used as described for the preparation of other native mAbs.

In another preferred embodiment of the invention, the recombinant mAbs of the invention are prepared by screening phage display libraries expressing recombinant mab-encoding polynucleotides (preferably encoding human recombinant mabs). See, for example, Ames et al., 1994, supra; Smith and Scott, Meth. Enzymol. 1993, 217:228; Kay et al., Gene, 1993, 128:59–65. This screening is carried out with the screening ligands described above for the preparation of native mAbs. The recombinant mAbs of the invention are prepared by subcloning the recombinant mAb-encoding polynucleotides into a suitable expression vector, expressing them in a suitable host (as described below), and isolating the recombinant mAbs.

The present invention provides recombinant expression vectors which include the synthetic, genomic, or cDNA-derived nucleic acid fragments of the invention, i.e. polynucleotides encoding the mabs of the invention. The nucleotide sequence coding for any of the mAbs of the invention can be inserted into an appropriate expression vector, i.e., a vector that contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translational signals can also be supplied by the native or source gene and/or its flanking regions.

A variety of host vector systems may be utilized to express the recombinant expression vectors of the invention. These include, but are not limited to, mammalian cell systems infected with recombinant virus (e.g., vaccinia virus, adenovirus, retroviruses, etc.); mammalian cell systems transfected with recombinant plasmids; insect cell systems infected with recombinant virus (e.g., baculovirus); microorganisms such as yeast containing yeast expression vectors, or bacteria transformed with recombinant bacteriophage DNA, recombinant plasmid DNA, or cosmid DNA (see, for example, Goeddel, 1990).

Useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well-known cloning vector pBR322 (American Type Culture Collection-"ATCC"-, 10801 University Boulevard, Manassas, Va. 20110-2209, United States of America; ATCC Accession No. 37017). These pBR322 "backbone sections," or functionally equivalent sequences, are combined with an appropriate promoter and the structural gene to be expressed. Promoters commonly used in recombinant microbial expression vectors include, but are not limited to, the lactose promoter system (Chang, et al., Nature 275:615), the tryptophan (trp) promoter (Goeddel, et al., 1980, Gene Expression Technology, Volume 185. Academic Press, Inc., San Diego, Calif.) and the tac promoter, or a fusion between the tac and trp promoters referred to as the trc promoter (Maniatis, 1982, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). Particularly preferred promoters include the T7 promoter, which is used in conjunction with host cell expression of a T7 RNA polymerase (see Studier et al. 1990, Meth. Enzymol. 185:60–89), and the trc promoter, which is found in several commercially available vectors, as described below.

Preferred bacterial expression vectors include, but are not limited to, the pET vectors (see Studier et al. 1990, supra) and the Trc vectors. Many of the pET vectors are commercially available from Stratagene Cloning Systems (La Jolla, Calif.). A particularly preferred vector is the pET Trc SO5/NI vector described below (SEQ ID NO:18). A Trc vector, pTrc 99A, is available from Pharmacia. Other Trc vectors include the pSE vectors (Invitrogen, San Diego, Calif.).

Preferred bacteria for expression of recombinant mAbs include *Bacillus subtilis* and, most preferably, *Escherichia coli*. A particularly preferred strain of *E. coli* is strain W3110 (ATCC designation 27325). Under certain unusual conditions it may be necessary to use standard bacterial genetics methods to prepare derivative strains of W3110, for example, when a contaminating bacteriophage ("phage") is present in the laboratory where the bacterial manipulations are being carried out. Generally, and particularly for large scale preparation of the recombinant anti-KSSKC mAbs of the invention, it is preferred to use unmodified W3110, or another fully characterized strain.

In cases where phage contamination is a problem and disinfection is not practicable or desirable, it is preferred to identify the phage contaminant and to then use a fully characterized bacterial strain having a known mutation rendering the bacterium resistant to the phage. Preferably the mutation is a null mutant for the receptor for the phage. In some instances, however, the generation use of a relatively uncharacterized phage-resistant derivative strain may be acceptable, particularly in small scale experimental work. When such derivative strains are desired, they may be prepared using the methods described below in Example 11.

For most purposes the use of unmodified W3110 or another fully characterized bacterial strain is generally preferred. This is particularly true for the preparation of phar-

maceutical agents comprising the recombinant anti-KSSKC mAbs of the invention. This is because of the problems, well known in the art, of using bacterial strains containing uncharacterized or partially characterized mutations for the production of ingredients of pharmaceutical agents.

The recombinant mAbs of the invention may also be expressed in fungal hosts, preferably yeast of the Saccharomyces genus such as *S. cerevisiae*. Fungi of other genera such as Aspergillus, Pichia or Kluyveromyces may also be employed. Fungal vectors will generally contain an origin of replication from the 2 μm yeast plasmid or another autonomously replicating sequence (ARS), a promoter, DNA encoding a mAb of the invention, sequences directing polyadenylation and transcription termination, and a selectable marker gene. Preferably, fungal vectors will include an origin of replication and selectable markers permitting transformation of both *E. coli* and fungi.

Suitable promoter systems in fungi include the promoters for metallothionein, 3-phosphoglycerate kinase, or other glycolytic enzymes such as enolase, hexokinase, pyruvate kinase, glucokinase, the glucose-repressible alcohol dehydrogenase promoter (ADH2), the constitutive promoter from the alcohol dehydrogenase gene, ADH1, and others. See, for example, Schena, et al. 1991 Meth. Enzymol. 194:389–398. Secretion signals, such as those directing the secretion of yeast alpha-factor or yeast invertase, can be incorporated into the fungal vector to promote secretion of a soluble recombinant mAb into the fungal growth medium. See Moir, et al., 1991, Meth. Enzymol. 194:491–507.

Preferred fungal expression vectors can be assembled using DNA sequences from pBR322 for selection and replication in bacteria, and fungal DNA sequences, including the ADH1 promoter and the alcohol dehydrogenase ADH1 termination sequence, as found in vector pAAH5 (Ammerer, 1983, Meth. Enzymol. 101:192). The ADH1 promoter is effective in yeast in that ADH1 mRNA is estimated to be 1–2% of total poly(A) RNA.

Various mammalian or insect cell culture systems can be employed to express recombinant mAbs. Suitable baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow, et al., 1988. Examples of suitable mammalian host cell lines include the COS cell of monkey kidney origin, mouse L cells, murine C127 mammary epithelial cells, mouse Balb/3T3 cells, Chinese hamster ovary cells (CHO), human 293 EBNA and HeLa cells, myeloma, and baby hamster kidney (BHK) cells, with myeloma cells, CHO cells, and human 293 EBNA cells being particularly preferred.

Mammalian expression vectors may comprise nontranscribed elements such as origin of replication, a suitable promoter and enhancer linked to the recombinant mAb gene to be expressed, and other 5' or 3' flanking sequences such as ribosome binding sites, a polyadenylation sequence, splice donor and acceptor sites, and transcriptional termination sequences.

The transcriptional and translational control sequences in mammalian expression vector systems to be used in transforming vertebrate cells may be provided by viral sources. For example, commonly used promoters and enhancers are derived from Polyoma virus, Adenovirus, Simian Virus 40 (SV40), and human cytomegalovirus, including the cytomegalovirus immediate-early gene 1 promoter and enhancer (CMV).

Particularly preferred eukaryotic vectors for the expression of recombinant anti-KSSKC mAbs are pAPEX-1 (SEQ ID NO:3 and, more preferably, pAPEX-3p, SEQ ID NO:4. The vector pAPEX-1 is a derivative of the vector pcDNAI/Amp (Invitrogen) which was modified to increase protein expression levels. First, the 3'-untranslated SV40 small-t antigen intron was removed by deletion of a 601 base pair XbaI/HpaI fragment since this intron is susceptible to aberrant splicing into upstream coding regions (Evans and Scarpulla, 1989 Gene 84:135; Huang and Gorman, 1990, Molec. Cell Biol. 10:1805). Second, a chimeric adenovirus-immunoglobulin hybrid intron was introduced into the 5'-untranslated region by replacing a 484 base pair NdeI-NotI fragment with a corresponding 845 base pair NdeI-NotI fragment from the vector pRc/CMV7SB (Sato et al., 1994, J. Biol. Chem. 269:17267). Finally, to increase plasmid DNA yields from *E. coli*, the resulting CMV promoter expression cassette was shuttled into the vector pGEM-4Z (Promega Corp. Madison, Wis.).

The vector pAPEX-3 is a derivative of the vector pDR2 (Clontech Laboratories, Inc. Palo Alto, Calif.) in which the EBNA gene was first removed by deletion of a 2.4 kb ClaI/AccI fragment. The RSV promoter was then replaced with the CMV promoter and the adenovirus/immunoglobulin chimeric intron by exchanging a 450 bp MluI/BamHI fragment from pDR2 with a 1.0 kb MluI/BamHI fragment from the vector pAPEX-1. For construction of pAPEX-3P, a 1.7 kb BstBI/SwaI fragment containing the HSV tk promoter and hygromycin phosphotransferase (hyg) gene was removed from pAPEX-3 and replaced with a 1.1 kb SnaBI/NheI fragment containing the SV40 early promoter and puromycin acetyltransferase (pac) gene (Morgenstern and Land, 1990, Nucleic Acids Res. 18:3587–3596) plus a 137 bp XbaI/ClaI fragment containing an SV40 polyadenylation signal from the vector pAPEX-1.

A particularly preferred host cell for the expression of recombinant mAb-encoding inserts in the pAPEX vectors is the human 293 EBNA cell line (Invitrogen, San Diego, Calif.).

Another preferred eukaryotic vector for the expression of recombinant mAbs is pcDNAI/Amp (Invitrogen Corporation, San Diego, Calif.). The pcDNAI/Amp expression vector contains the human cytomegalovirus immediate-early gene I promoter and enhancer elements, the Simian Virus 40 (SV40) consensus intron donor and acceptor splice sequences, and the SV40 consensus polyadenylation signal. This vector also contains an SV40 origin of replication that allows for episomal amplification in cells (e.g., COS cells, MOP8 cells, etc.) transformed with SV40 large T antigen, and an ampicillin resistance gene for propagation and selection in bacterial hosts.

Purified recombinant mAbs are prepared by culturing suitable host/vector systems to express the recombinant mAb translation products of the nucleic acid molecules of the present invention, which are then purified from the culture media or cell extracts of the host system, e.g., the bacteria, insect cells, fungal, or mammalian cells. Fermentation of fungi or mammalian cells that express recombinant mAb proteins containing a histidine tag sequence (a sequence comprising a stretch of at least 5 histidine residues) as a secreted product greatly simplifies purification. Such a histidine tag sequence enables binding under specific conditions to metals such as nickel, and thereby to nickel (or other metal) columns for purification. Recombinant mAbs may also be purified by protein G affinity chromatography (Proudfoot et al., 1992, Protein Express. Purif. 3:368).

Additional preferred embodiments are numbered and set forth below as "favored embodiments."

FAVORED EMBODIMENTS

1. A method for the treatment of glomerulonephritis in a patient in need of such treatment comprising introducing an

antibody that binds to complement component C5 into the patient's bloodstream in an amount effective to substantially reduce the cell-lysing ability of complement present in the patient's blood.

2. The method of favored embodiment 1 wherein the antibody reduces the conversion of complement component C5 into complement components C5a and C5b.

3. The method of favored embodiment 1 wherein the antibody binds to C5b.

4. The method of favored embodiment 1 wherein the antibody does not substantially inhibit formation of complement component C3b.

5. The method of favored embodiment 1 wherein the antibody is introduced into the patient's bloodstream in a dose that is not greater than 0.1 grams per kilogram.

6. An article of manufacture comprising packaging material and a pharmaceutical agent contained within said packaging material, wherein:

(a) said pharmaceutical agent comprises an antibody to complement component C5, said antibody being effective in substantially reducing the cell-lysing ability of complement present in the patient's blood; and

(b) said packaging material comprises a label which indicates that said pharmaceutical agent is for use in the treatment of kidney disease.

7. The article of manufacture of favored embodiment 6 wherein the label indicates that said pharmaceutical agent is for use in the treatment of nephritis.

8. The article of manufacture of favored embodiment 7 wherein the label indicates that said pharmaceutical agent is for use in the treatment of glomerulonephritis.

9. The article of manufacture of favored embodiment 6 wherein the pharmaceutical agent is to be used at a dosage level not greater than 0.1 grams per kilogram.

10. An antibody comprising at least one antibody-antigen binding site, said antibody exhibiting specific binding to human complement component C5, said specific binding being targeted to the alpha chain of human complement component C5, wherein the antibody inhibits complement activation in a human body fluid and does not specifically bind to the human complement activation product free C5a.

11. The antibody of favored embodiment 10 wherein the inhibition of complement activation in the human body fluid is measurable as a substantial increment of blockade of C5a generation and a substantial increment of blockade of complement hemolytic activity in the body fluid, said increment of blockade of C5a generation being substantially equal to said increment of blockade of complement hemolytic activity.

12. The antibody of favored embodiment 10 wherein, upon binding to human C5, the antibody substantially inhibits the ability of C5 to bind to human complement component C3.

13. The antibody of favored embodiment 10 wherein, upon binding to human C5, the antibody substantially inhibits the ability of C5 to bind to human complement component C4.

14. The antibody of favored embodiment 10 wherein the antibody binds specifically with a 5G46k fragment.

15. The antibody of favored embodiment 10 wherein the antibody binds specifically to a 5G27k fragment.

16. The antibody of favored embodiment 10 wherein the antibody binds specifically to a 5G325aa peptide.

17. The antibody of favored embodiment 10 wherein the antibody binds specifically to a 5G200aa peptide.

18. The antibody of favored embodiment 10 wherein the antibody binds specifically to a KSSKC peptide.

19. The antibody of favored embodiment 10 wherein the inhibition of complement activation in the human body fluid is measurable as a substantially complete blockade of C5a generation in the body fluid and a substantially complete blockade of complement hemolytic activity in the body fluid when the antibody is added to the body fluid at a concentration yielding a ratio equal to or less than 10 moles of antibody-antigen binding sites of the antibody to 1 mole of human C5 in the body fluid.

20. The antibody of favored embodiment 19 wherein the concentration yields a ratio equal to or less than 3 moles of antibody-antigen binding sites of the antibody to 1 mole of human C5 in the body fluid.

21. Hybridoma 5G1.1 having ATCC designation HB-11625.

22. An antibody produced by the hybridoma of favored embodiment 21.

23. An antibody that can compete with the antibody of favored embodiment 22 for binding to the alpha chain of human C5.

24. A nucleic acid molecule comprising a nucleotide sequence encoding an scFv polypeptide comprising an amino acid sequence corresponding to amino acid 1 through amino acid 248 of SEQ ID NO:7.

25. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a variable light chain region amino acid sequence corresponding to amino acid 3 through amino acid 110 of SEQ ID NO:9.

26. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:10.

27. An isolated protein comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 3 through amino acid 110 of SEQ ID NO:9.; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:10.

28. An isolated polypeptide comprising an amino acid sequence encoded by the nucleic acid molecule of favored embodiment 24, favored embodiment 25, or favored embodiment 26, wherein the polypeptide is an antibody.

29. A nucleic acid vector comprising a first nucleic acid molecule covalently and operatively linked to a second nucleic acid molecule so that a host containing the vector expresses the polypeptide coded for by the first nucleic acid molecule, wherein the first nucleic acid molecule is the nucleic acid molecule of favored embodiment 24, favored embodiment 25, or favored embodiment 26.

30. A recombinant host cell containing the nucleic acid vector of favored embodiment 29.

31. A method for producing an isolated C5 antibody polypeptide comprising growing the recombinant host cell of favored embodiment 30 such that the polypeptide encoded by the first nucleic acid molecule of the vector is expressed by the host cell, and isolating the expressed polypeptide, wherein the expressed polypeptide is an anti-C5 antibody.

32. The isolated anti-C5 antibody of favored embodiment 31.

33. A nucleic acid molecule comprising a nucleotide sequence encoding an scFv comprising an amino acid sequence corresponding to amino acid 1 through amino acid 248 of SEQ ID NO:8.

34. A nucleic acid molecule comprising a nucleotide sequence encoding an scFv comprising an amino acid sequence corresponding to amino acid 1 through amino acid 248 of SEQ ID NO:17.

35. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a variable light chain region amino acid sequence corresponding to amino acid 1 through amino acid 108 of SEQ ID NO:15.

36. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a variable light chain region amino acid sequence corresponding to amino acid 3 through amino acid 110 of SEQ ID NO:14.

37. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:16.

38. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:12.

39. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:11.

40. An isolated protein comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 1 through amino acid 108 of SEQ ID NO:15; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:16.

41. An isolated protein comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 1 through amino acid 1 through amino acid 108 of SEQ ID NO:15; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:12.

42. An isolated protein comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 1 through amino acid 108 of SEQ ID NO:15; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:11.

43. An isolated protein comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 3 through amino acid 110 of SEQ ID NO:14; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:16.

44. An isolated protein comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 3 through amino acid 110 of SEQ ID NO:14; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:12.

45. An isolated protein comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 3 through amino acid 110 of SEQ ID NO:14; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:11.

46. An isolated protein comprising the amino acid sequence encoded by the nucleic acid molecule of favored embodiment 33, favored embodiment 34, favored embodiment 35, favored embodiment 36, favored embodiment 37, favored embodiment 38, or favored embodiment 39, wherein the isolated protein is an anti-C5 antibody.

47. A nucleic acid vector comprising a first nucleic acid molecule covalently and operatively linked to a second nucleic acid molecule so that a host containing the vector expresses the polypeptide coded for by the first nucleic acid molecule, wherein the first nucleic acid molecule is the nucleic acid molecule of favored embodiment 33, favored embodiment 34, favored embodiment 35, favored embodiment 36, favored embodiment 37, favored embodiment 38, or favored embodiment 39.

48. A recombinant host cell containing the nucleic acid vector of favored embodiment 47.

49. A method for producing an isolated anti-C5 antibody protein comprising growing the recombinant host cell of favored embodiment 48 such that a protein encoded by the nucleic acid molecule is expressed by the host cell, and isolating the expressed protein, wherein the expressed protein is an anti-C5 antibody.

50. The isolated anti-C5 antibody of favored embodiment 47.

51. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable light region CDR3 comprising an amino acid sequence corresponding to amino acid 93 through amino acid 98 of SEQ ID NO:7;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

52. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable light region CDR3 comprising an amino acid sequence corresponding to amino acid 91 through amino acid 99 of SEQ ID NO:8;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

53. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid 156 through amino acid 159 of SEQ ID NO:7;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

54. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid 152 through amino acid 161 of SEQ ID NO:8;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

55. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable heavy region CDR2 comprising an amino acid sequence cor-

responding to amino acid 179 through amino acid 182 of SEQ ID NO:7;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

56. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid 176 through amino acid 186 of SEQ ID NO:8;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

57. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable heavy region CDR3 comprising an amino acid sequence corresponding to amino acid 226 through amino acid 236 of SEQ ID NO:7;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

58. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable heavy region CDR3 comprising an amino acid sequence corresponding to amino acid 225 through amino acid 237 of SEQ ID NO:8;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

59. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable light region CDR3 comprising an amino acid sequence corresponding to amino acid 91 through amino acid 99 of SEQ ID NO:8;

(b) a nucleotide sequence encoding a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid 152 through amino acid 161 of SEQ ID NO:8;

(c) a nucleotide sequence encoding a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid 176 through amino acid 186 of SEQ ID NO:8; and

(d) a nucleotide sequence encoding a variable heavy region CDR3 comprising an amino acid sequence corresponding to amino acid 225 through amino acid 237 of SEQ ID NO:8.

60. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable light region CDR3 comprising an amino acid sequence corresponding to amino acid 91 through amino acid 99 of SEQ ID NO:8;

(b) a nucleotide sequence encoding a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid 152 through amino acid 161 of SEQ ID NO:8;

(c) a nucleotide sequence encoding a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid 176 through amino acid 192 of SEQ ID NO:8; and

(d) a nucleotide sequence encoding a variable heavy region CDR3 comprising an amino acid sequence corresponding to amino acid 225 through amino acid 237 of SEQ ID NO:8.

61. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable light region CDR3 comprising an amino acid sequence corresponding to amino acid 91 through amino acid 99 of SEQ ID NO:8;

(b) a nucleotide sequence encoding a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid 152 through amino acid 161 of SEQ ID NO:8;

(c) a nucleotide sequence encoding a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid 179 through amino acid 182 of SEQ ID NO:7; and

(d) a nucleotide sequence encoding a variable heavy region CDR3 comprising an amino acid sequence corresponding to amino acid 225 through amino acid 237 of SEQ ID NO:8.

62. An isolated protein comprising the amino acid sequence encoded by the nucleic acid molecule of favored embodiment 51, favored embodiment 52, favored embodiment 53, favored embodiment 54, favored embodiment 55, favored embodiment 56, favored embodiment 57, favored embodiment 58, favored embodiment 59, favored embodiment 60 or favored embodiment 87.

63. The isolated protein of favored embodiment 62 wherein the protein is an anti-C5 antibody.

64. A nucleic acid vector comprising a first nucleic acid molecule, said first nucleic acid molecule corresponding to the nucleic acid molecule of favored embodiment 51, favored embodiment 52, favored embodiment 53, favored embodiment 54, favored embodiment 55, favored embodiment 56, favored embodiment 57, favored embodiment 58, or favored embodiment 87 covalently and operatively linked to a second nucleic acid molecule so that a host containing the vector expresses the protein encoded by the first nucleic acid molecule.

65. A recombinant host cell containing the nucleic acid vector of favored embodiment 64.

66. A method for producing an anti-C5 antibody comprising growing the recombinant host cell of favored embodiment 65 so that the protein encoded by the nucleic acid molecule is expressed by the host cell, and isolating the expressed protein, wherein the expressed protein is an anti-C5 antibody.

67. The anti-C5 antibody of favored embodiment 66.

68. An isolated 5G46k fragment of human complement component C5.

69. An isolated 5G27k fragment of human complement component C5.

70. An isolated 5G325aa peptide.

71. An isolated 5G200aa peptide.

72. An isolated oligopeptide comprising an amino acid sequence corresponding to amino acid 8 through amino acid 12 of SEQ ID NO:1, i.e., Lys Ser Ser Lys Cys, or in single letter notation, KSSKC.

73. A method of inducing an animal to produce an anti-C5 antibody comprising repeatedly immunizing an animal with the isolated alpha chain of human C5.

74. A method of inducing an animal to produce an anti-C5 antibody comprising immunizing an animal with the isolated 5G46k fragment of favored embodiment 68.

75. A method of inducing an animal to produce an anti-C5 antibody comprising immunizing an animal with the isolated 5G27k fragment of favored embodiment 69.

76. A method of inducing an animal to produce an anti-C5 antibody comprising immunizing an animal with the isolated 5G325aa peptide of favored embodiment 70.

77. A method of inducing an animal to produce an anti-C5 antibody comprising immunizing an animal with the isolated 5G200aa peptide of favored embodiment 71.

78. A method of inducing an animal to produce an anti-C5 antibody comprising immunizing an animal with the isolated oligopeptide of favored embodiment 72.

79. A method of identifying an anti-C5 antibody comprising screening candidate antibodies with the isolated alpha chain of human C5.

80. A method of identifying an anti-C5 antibody comprising screening candidate antibodies with the isolated 5G46k fragment of favored embodiment 68.

81. A method of identifying an anti-C5 antibody comprising screening candidate antibodies with the isolated 5G27k fragment of favored embodiment 69.

82. A method of identifying an anti-C5 antibody comprising screening candidate antibodies with the isolated 5G325aa peptide of favored embodiment 70.

83. A method of identifying an anti-C5 antibody comprising screening candidate antibodies with the isolated 5G200aa peptide of favored embodiment 71.

84. A method of identifying an anti-C5 antibody comprising screening candidate antibodies with the isolated oligopeptide of favored embodiment 72.

85. A method of treating a patient in need of complement inhibition comprising administering the antibody of favored embodiment 10, favored embodiment 22, favored embodiment 23, favored embodiment 28, favored embodiment 32, favored embodiment 46, favored embodiment 50, favored embodiment 63, or favored embodiment 67 to the patient in an amount effective to substantially reduce hemolytic activity in a body fluid of the patient.

86. The antibody of favored embodiment 10 wherein the antibody is a recombinant antibody that comprises a human constant domain.

87. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid 176 through amino acid 192 of SEQ ID NO:8;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

88. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid 176 through amino acid 192 of SEQ ID NO:8;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

89. An isolated antibody comprising any one of the CDR regions of CO12, CO13, CO14, Co15, DO12b, DO12C, DO12D.

90. An isolated nucleic acid molecule encoding the antibody of favored embodiment 89.

EXAMPLES

Without intending to limit it in any manner, the present invention will be more fully described by the following examples. The methods and materials which are common to various of the examples are as follows.

Induction of GN in Mice

Four month old female B10.D2/nSnJ mice averaging approximately 25 gms each were obtained from the Jackson Laboratory, Bar Harbor, Me. Mice were injected with 0.1 mL daily (six days per week) of a 40 mg/mL solution of horse apoferritin (HAF), which was prepared by dilution of a saline solution of HAF (Sigma Chemical Company Catalog No. A-3641) with PBS.

Anti-C5 Monoclonal Antibodies

Monoclonal antibodies that bind to complement component C5 of the mouse were prepared by standard methods as an IgG fraction from supernatants of cultures of hybridoma BB5.1 (Frei, et al., 1987), which was obtained from Dr. Brigitta Stockinger of the National Institute for Medical Research, Mill Hill, London, England.

Histology

Kidneys were subjected to microscopic analysis using standard histochemical staining and immunofluorescence techniques. Periodic Acid Schiff (PAS) staining of 5 paraffin sections was by standard methods using a HARLECO PAS histochemical reaction set (EM Diagnostic Systems, Gibbstown, N.J., number 64945/93) according to the manufacturer's directions.

Immunofluorescence staining of 5μ cryostat sections was carried out by standard methods using FITC conjugated sheep anti-mouse C3 (Biodesign International, Kennebunk, Me, Catalog No. W90280F) to detect murine complement component C3, or FITC conjugated goat anti-mouse IgG, IgA, and IgM (Zymed Laboratories, South San Francisco, Calif., Catalog No. 65-6411) to detect immune complexes.

Urine Assays

Protein and glucose levels were determined by spotting urine samples on CHEMSTRIP 2GP dipsticks (Boehringer Mannheim Diagnostics, Indianapolis, Ind., Catalog No. 200743). The detection areas of these strips change color when exposed to urine containing protein or glucose; a lack of color change indicates no detectable protein or glucose is present. The level of analyte in the urine being tested is read out by matching changed colors with color charts supplied by the manufacturer. The urine protein chart shows colors corresponding to trace, 30, 100, and 500 mg/dL.

Cell Lysis Assays

The cell-lysing ability of complement in blood can be determined using hemolytic assays that are performed as follows: Chicken erythrocytes are washed well in GVBS (Rollins, et al., *J. Immunol* 144:3478–3483, 1990, Sigma Chemical Co. St. Louis, Mo., catalog No. G-6514) and resuspended to 2×10^8/mL in GVBS. Anti-chicken erythrocyte antibody (IgG fraction of anti-chicken-RBC antiserum, Intercell Technologies, Hopewell, N.J.) is added to the cells at a final concentration of 25 μg/mL and the cells are incubated for 15 min. at 23° C. The cells are washed 2× with GVBS and 5×10^6 cells are resuspended to 30 μL in GVBS. A 100 μL volume of serum test solution is then added to yield a final reaction mixture volume of 130 μL. As used herein, reference to the serum percentage and/or serum input in these assays indicates the percent serum in the 100 μL volume of serum test solution.

For assays of mouse serum activity, the 100 μL volume of serum test solution contained 50 μL of diluted (in GVBS) mouse serum and 50 μL of human C5 deficient serum (Quidel Corporation, San Diego, Calif.). For assays of human serum activity, the serum test solution may contain up to 100% human plasma or serum, with hybridoma supernatants and/or GVBS being added to yield the 100 μL volume. For the assays used to screen hybridoma supernatants discussed below in Example 7, each 100 μL volume of serum test solution contained 50 μL of hybridoma supernatant and 50 μL of a 10% solution of human serum in GVBS, yielding a 5% human serum input.

After incubation for 30 min. at 37° C., percent hemolysis was calculated relative to a fully lysed control sample. Hemolysis was determined by spinning the cells down and measuring released hemoglobin in the supernatant as the optical density at 415 nm.

A 50% reduction in hemolysis after treatment with the anti-C5 antibodies used in the practice of the invention means that the percent hemolysis after treatment is one half of the percent hemolysis before treatment.

Example 1

Anti-C5 Antibodies Inhibit Glomerular Inflammation and Enlargement

This example illustrates that anti-C5 antibodies will inhibit glomerular inflammation and enlargement.

The protocol for these experiments was as follows. GN-induced mice were treated with anti-C5 antibodies or with PBS as a control after 2 weeks of GN induction. Each mouse received 750 μg of anti-C5 monoclonal antibodies in PBS (30 mg/kg in a 25 gm mouse) or an equal volume of PBS alone. The amount injected was from 0.25 to 0.365 mL (the concentration of antibodies in PBS varied), which was administered by intraperitoneal injection once a day, six days a week. After an additional 2 weeks of induction and treatment, the animals were sacrificed and kidneys were harvested and prepared for histological examination as described above. Kidneys were also obtained from age-matched uninduced and untreated control mice.

FIG. 1 shows sections of mouse kidneys with a single glomerulus located centrally amidst surrounding interstitium and cross sections of convoluted tubules in each section. As can be seen therein, the kidneys of the GN-induced, PBS-treated mice (FIG. 1B) developed severe crescentic glomerular pathology, including inflammatory glomerular hypercellularity, apparent basement membrane thickening, and glomerular enlargement, while the glomeruli of the GN-induced, anti-C5-treated animals (FIG. 1C) were essentially indistinguishable from the glomeruli of the normal healthy kidneys of the uninduced untreated mice (FIG. 1A).

Note that in the glomeruli with severe crescentic pathology, the size of the glomerular capillary network (glomerular tuft) is not enlarged, but shows signs of compression by a crescentic-shaped proliferation of epithelial cells and PAS-positive material, and the Bowman's capsule is dramatically enlarged. Also note that in the section of diseased glomerulus shown in FIG. 1B, the capillary network is split in half by a projection of the hypercellular crescentic mass.

The non-inflamed glomerulus of the uninduced untreated mouse shown in FIG. 1A is approximately 100μ in diameter; the inflamed glomerulus of the GN-induced, PBS treated mouse shown in FIG. 1B is approximately 175μ in diameter; the non-inflamed glomerulus of the GN-induced, anti-C5-treated mouse shown in FIG. 1C is approximately 90μ in diameter.

Example 2

Anti-C5 Antibodies Prevent/Reduce Proteinuria Associated with GN

This example demonstrates that treatment with anti-C5 antibodies results in the prevention/reduction of kidney damage as evidenced by the lack of significant amounts of protein in the urine (i.e. the presence of less than 100 mg/dL of protein in the urine).

The protocol for the experiments of this example was the same as that used in the experiments of Example 1. Five PBS-treated, GN-induced mice, 6 anti-C5-treated, GN-induced mice, and 4 age-matched untreated uninduced mice were used in this study. A first set of urine samples was analyzed prior to treatment after the initial 2 week induction period. A second set of urine samples was analyzed after the 2 week treatment period. None of the untreated uninduced control animals had detectable protein in their urine at either of these timepoints.

The results obtained with the GN-induced mice are set forth in Table 1. As shown therein, at the end of the 2 week PBS treatment period, 4 out of the 5 PBS treated (control) animals developed significant proteinuria, i.e., at least 100 mg/dL of protein in the urine. The fifth animal (mouse D in Table 1) did not have detectable protein in the urine at either timepoint but, unlike the other mice in the study, was found to have very high levels of glucose in the urine after the 2 week PBS treatment period, suggesting that this animal was physiologically compromised.

In the anti-C5-treated, GN-induced group, the one mouse that developed significant proteinuria at the end of the initial 2 week induction period (mouse 6 in Table 1) improved by the end of the 2 week antibody treatment period. In addition, in contrast to the development of significant proteinuria in 4 out of 5 PBS-treated, GN-induced mice, none of the anti-C5-treated, GN-induced mice exhibited significant proteinuria at the end of the 2 week antibody treatment period.

Example 3

Anti-C5 Antibodies do not Inhibit Glomerular Immune Complex Deposition

This example demonstrates that anti-C5 antibodies used in the practice of the invention achieve their therapeutic effects even though immune complexes are deposited in the glomeruli of treated animals at equivalent levels to those seen in the glomeruli of PBS-treated animals. The example further illustrates that the mechanism of operation of the anti-C5 antibodies is not through the inhibition of immune complex deposition in the glomerulus.

The protocol used in the experiments of this example was the same as that used in the experiments of Example 1. Immunofluorescence staining as described above was performed on sections from the same kidneys harvested in Example 1.

The results are shown in FIG. 2. As can be seen in this figure, equivalent amounts of immune complexes were deposited in the glomeruli of the kidneys of both the PBS-treated, GN-induced mice (FIG. 2B) and the anti-C5-treated, GN-induced mice (FIG. 2C), but not in the untreated uninduced controls (FIG. 2A). Kidneys of GN-induced mice harvested after the 2 week induction period, but before treatment, showed immune complex deposits in the glomeruli, but at lower levels (as indicated by lower fluorescence intensity) than in the kidney sections shown in FIG. 2B and FIG. 2C.

Example 4

Anti-C5 Antibodies Inhibit C5b-9 Generation

This example demonstrates that the anti-C5 antibodies used in the practice of the invention inhibit C5b-9 generation. C5b-9 generation was assayed in 2 ways: (1) by testing the cell-lysing (hemolytic) ability of blood samples, and (2) by measuring levels of soluble C5b-9 in blood samples.

FIG. 3 shows the results of cell lysis assays performed as described above, with mouse serum added to the percentage indicated on the X axis ("serum input %"). In these assays, serum from GN-induced animals treated with either anti-C5 antibodies in PBS or PBS alone (see above) was assayed at the end of the two week treatment period. Serum from normal, uninduced, uninjected mice ("normal mouse serum") obtained from Sigma Chemical Company (St. Louis, Mo., Catalog No. S-3269) was also assayed as an additional control. These results indicate that the anti-C5

monoclonal antibody administered to mice at a dosage of 30 mg/Kg completely blocked the cell lysing ability of mouse blood at serum input levels 4-fold higher than the levels of normal serum that produce maximum hemolysis in the assay.

The effects of an anti-C5 monoclonal antibody raised to human C5 was evaluated in circulating human blood. Hybridoma N19/8 (Wurzner, et al., 1991) was obtained from Dr. Otto Götze, Department of Immunology, University of G öttingen, FRG. The C5 monoclonal antibody was prepared following immunization of mice with purified human C5 protein as described in Wurzner, et al., (1991). The hybridoma was propagated in mice, and the monoclonal antibody recovered and purified as an IgG fraction from mouse ascites fluid (*Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory; New York, 1988; *Current Protocols In Immunology*, John Wiley & Sons, New York, 1992).

To carry out these experiments, as well as others described below in Examples 5 and 6, 300 mL of whole human blood was drawn from a healthy human donor and additionally a 1 mL sample was removed as a control sample for later analysis. The blood was diluted to 600 mL by the addition of Ringer's lactate solution containing 10 U/mL heparin. The anti-C5 mAb (30 mg in sterile PBS) was added to the diluted blood to a final concentration of 50 μg/mL (results using test samples obtained in this way are labeled "+anti-C5 sample" in FIG. 4 and FIG. 6). In a control experiment, an equal volume of sterile PBS was added to diluted blood (results using control samples obtained in this way are labeled "−anti-C5 sample" in FIG. 4 and FIG. 6).

The blood was then used to prime the extracorporeal circuit of a COBE CML EXCEL membrane oxygenator cardiopulmonary bypass (CPB) machine (Cobe BCT, Inc., Lakewood, Colo.) and circulation through the circuit was started. The circuit was cooled to 28° C. and circulated for 60 minutes. The circuit was then warmed to 37° C. and circulated for an additional 30 minutes, after which time the experiment was terminated. Mechanical circulation of blood in this fashion activates the complement cascade. Samples were taken at several time points.

At each time point an aliquot of blood was taken, and subaliquots were centrifuged to remove all cells and the remaining plasma diluted 1:1 in QUIDEL sample preservation solution (Quidel Corporation, San Diego, Calif.) and stored at −80° C. for subsequent evaluation of soluble C5b-9 (sC5b-9) generation. Diluted subaliquots of plasma were also frozen for evaluation of C3a generation (see Example 5, below). Undiluted subaliquots of plasma were frozen at −80° C. for analysis in hemolytic assays to evaluate the pharmacokinetics of the effects of the anti-C5 antibodies on the cell lysing ability of complement present in the blood (see Example 6, below). These experiments are also discussed in copending U.S. patent application Ser. No. 08/217,391, filed Mar. 23, 1994, now U.S. Pat. No. 5,853,722.

sC5b-9 assays were performed before the addition of the antibody or the commencement of the CPB circuit (labeled "Pre Tx" in FIG. 4 and FIG. 6) using undiluted blood (i.e. blood from the 1 mL sample taken before the blood was diluted with Ringer's lactate solution—labeled "undil" in FIG. 4 and FIG. 6) and Ringer's lactate solution diluted blood (labeled "dil" in FIG. 4 and FIG. 6). Samples of Ringer's lactate solution diluted blood to which the antibody had been added (labeled "Post Tx" in FIG. 4 and FIG. 6) were assayed at the times indicated after starting the CPB circuit.

As can be seen in FIG. 4, while sC5b-9 levels were more than 4-fold higher in untreated samples after 90 minutes of circulation than before circulation, the anti-C5 antibody completely inhibited C5b-9 generation throughout the 90 minute time course of circulation so that sC5b-9 levels during circulation were essentially equivalent to control, uncirculated samples, at all timepoints.

Example 5

Anti-C5 Antibodies do not Inhibit C3 Deposition or Activation

This example demonstrates that treatment with anti-C5 antibodies does not result in the inhibition of the activation of complement component C3 or in the deposition of C3 or its activated fragments in glomeruli.

The deposition of C3, or the fragments generated by its activation (e.g., C3a and C3b), in the glomeruli of GN-induced and GN-uninduced mice was visualized by immunofluorescence staining with a FITC-conjugated sheep anti-mouse C3 antibody preparation using standard methods, as described above. As can be seen in FIG. 5, kidneys of the PBS-treated (FIG. 5B) and the anti-C5 antibody-treated (FIG. 5C) GN-induced mice had roughly equivalent levels of C3 immunoreactive material in the glomeruli, while the uninduced untreated control mice had only traces of C3 immunoreactive material in their kidneys (FIG. 5A).

Note that the print shown in FIG. 5A was overexposed compared to those of FIG. 5B and FIG. 5C to show the very slight levels of reactivity present in normal uninduced kidneys. Kidneys of GN-induced mice harvested after the 2 week induction period, but before treatment, showed C3 immunoreactive materials in the glomeruli, but at lower levels (as indicated by lower fluorescence intensity) than in the kidney sections shown in FIG. 5B and FIG. 5C.

Anti-human C5 antibodies were also tested for possible inhibition of C3 activation in human blood prepared and circulated as described above in Example 4. Activation of complement component C3 was indicated by the presence in the blood of the C3 activation product C3a. C3a assays were performed as follows.

The plasma samples that had previously been diluted in QUIDEL sample preservation solution and frozen (see Example 4) were assayed for the presence of C3a by using the QUIDEL C3A EIA kit (Quidel Corporation, San Diego, Calif.) according to the manufacturer's specifications. Concentrations of C3a in the samples is expressed as ng/well as determined by comparison to a standard curve generated from samples containing known amounts of human C3a.

As seen in FIG. 6, the addition of the anti-C5 mAb had no inhibitory effect on the production of C3a during the circulation of human blood in this experiment.

Example 6

Pharmacokinetics of Anti-C5 Antibodies

The in vivo duration of action of mAb BB5.1, and a Fab' fragment of mAb BB5.1 (prepared by standard methods) was determined in normal female BALB/cByJ mice (averaging approximately 20 gms each) which were obtained from the Jackson Laboratory, Bar Harbor, Me. The mice were given a single intravenous injection (at 35 mg/kg body weight) of the mAB or the Fab' fragment of the mAb (or an equal volume of PBS as a control). Blood samples were collected from the retroorbital plexus at 1, 4, 24, 96, and 144 hours after administration of PBS; 4, 16, and 24 hours after administration of the Fab' fragment of mAb

BB5.1; and 4, 24, 48, 72, 96, and 144 hours after administration of intact mAb BB5.1.

FIG. 7A shows the time course of inhibition of the cell-lysing ability of complement in mouse blood (determined, by testing serum obtained from the blood and diluted to 2.5%, as described above) after the in vivo administration of the mAb, the Fab' fragment, or the PBS. As shown in the figure, the mAb almost completely inhibited the hemolytic activity of the blood throughout the 6 day test period. The Fab', however, had a half-life of approximately 24 hours.

In addition to the above experiments, at the end of the 6 day testing period all of the mice were sacrificed. Kidneys, lungs, and livers were harvested and examined by gross inspection, as well as by microscopic examination of stained sections. All of the organs of the anti-C5 antibody treated animals appeared the same as those taken from PBS control treated animals. The overall appearance of the test and control mice was also indistinguishable prior to necropsy.

Anti-human C5 antibodies were also tested for pharmacokinetic properties in circulating human blood as described above in Example 4. As described therein, the hemolysis inhibiting effects of an anti-human C5 monoclonal antibody were assayed over a 90 minute period of circulation. The results of these assays are charted in FIG. 7B, and show that the N19/8 anti-C5 mAb essentially completely inhibited the cell lysing ability of the human blood during the entire 90 minute period of circulation.

The results of these experiments demonstrate that the anti-C5 antibodies will survive in the bloodstream for a substantial period of time, thus making periodic administration practical.

Example 7

Preparation of Anti-C5 Monoclonal Antibodies

A monoclonal antibody suitable for use in the practice of the present invention was prepared in accordance with the teachings of Sims, et al., U.S. Pat. No. 5,135,916, as follows.

Balb/c mice were immunized three times by intraperitoneal injection with human C5 protein (Quidel Corporation, San Diego, Calif., Cat # A403). The first injection contained 100 μg of C5 protein in a complete Freund's adjuvant emulsion, the second immunization contained 100 μg of C5 protein in an incomplete Freund's adjuvant emulsion, and the third immunization was 100 μg of protein in PBS. The mice were injected at roughly 2 month intervals.

Fusions of splenocytes to myeloma cells to generate hybridomas were performed essentially as described in Current Protocols in Immunology (John Wiley & Sons, New York, 1992, pages 2.5.1 to 2.5.17). One day prior to fusion the mice were boosted IV with 100 μg of C5 protein. On the day of fusion, the immunized mice were sacrificed and spleens was harvested. SP2/0-AG14 myeloma cells (ATCC CRL#1581) were used as the fusion partner. SP2/0-AG14 cultures were split on the day before the fusion to induce active cell division. A ratio of 1:10 (myeloma cells:splenocytes) was used in the fusions.

The cells were fused using PEG 1450 in PBS without calcium (Sigma Chemical Company, St. Louis, Mo., Catalog No. P-7181) and plated at 1–2.5x10^5 cells per well. Selection in EX-CELL 300 medium (JRH Biosciences, Lexena, Kans., Catalog No. 14337-78P) supplemented with 10% heat inactivated fetal bovine serum (FBS); glutamine, penicillin and streptomycin (GPS); and HAT (Sigma Chemical Company, St. Louis, Mo., Catalog No. H-0262) was started the following day. The fusions were then fed every other day with fresh FBS, GPS, and HAT supplemented medium. Cell death could be seen as early as 2 days and viable cell clusters could be seen as early as 5 days after initiating selection. After two weeks of selection in HAT, surviving hybridomas chosen for further study were transferred to EX-CELL 300 medium supplemented with FBS, GPS, and HT (Sigma Chemical Company, St. Louis, Mo., Catalog No. H-0137) for 1 week and then cultured in EX-CELL 300 medium supplemented with FBS and GPS.

Hybridomas were screened for reactivity to C5 and inhibition of complement-mediated hemolysis 10–14 days after fusion, and were carried at least until the screening results were analyzed. The screen for inhibition of hemolysis was the chicken erythrocyte lysis assay described above. The screen for C5 reactivity was an ELISA, which was carried out using the following protocol:

A 50 μL aliquot of a 2 μg/mL solution of C5 (Quidel Corporation, San Diego, Calif.) in sodium carbonate /bicarbonate buffer, pH 9.5, was incubated overnight at 4° C. in each test well of a 96 well plate (NUNC-IMMUNO F96 POLYSORP, A/S Nunc, Roskilde, Denmark). The wells were then subjected to a wash step. (Each wash step consisted of three washes with TBST.) Next, test wells were blocked with 200 μL of blocking solution, 1% BSA in TBS (BSA/TBS) for 1 hour at 37° C. After an additional wash step, a 50 μL aliquot of hybridoma supernatant was incubated in each test well for 1 hour at 37° C. with a subsequent wash step. As a secondary (detection) antibody, 50 μL of a 1:2000 dilution of horseradish peroxidase (HRP) conjugated goat anti-mouse IgG in BSA/TBS, was incubated in each test well for 1 hour at 37° C., followed by a wash step. Following the manufacturer's procedures, 10 mg of O-phenylenediamine (Sigma Chemical Company, St. Louis, Mo., Catalog No. P-8287) was dissolved in 25 mLs of phosphate-citrate buffer (Sigma Chemical Company, St. Louis, Mo., Catalog No. P-4922), and 50 μL of this substrate solution was added to each well to allow detection of peroxidase activity. Finally, to stop the peroxidase detection reaction, a 50 μL aliquot of 3N hydrochloric acid was added to each well. The presence of antibodies reactive with C5 in the hybridoma supernatants was read out by a spectrophotometric OD determination at 490 nm.

The supernatant from a hybridoma designated as 5G1.1 tested positive by ELISA and substantially reduced the cell-lysing ability of complement present in normal human blood in the chicken erythrocyte hemolysis assay. Further analyses revealed that the 5G1.1 antibody reduces the cell-lysing ability of complement present in normal human blood so efficiently that, even when present at roughly one-half the molar concentration of human C5 in the hemolytic assay, it can almost completely neutralize serum hemolytic activity.

Immunoblot analysis was undertaken to further characterize the 5G1.1 mAb. Human C5 (Quidel Corporation, San Diego, Calif., Catalog No. A403) was subjected to polyacrylamide gel electrophoresis under reducing conditions, transferred to a nitrocellulose membrane, and probed with the 5G1.1 mAb as a purified IgG preparation. Two bands were immunoreactive with the 5G1.1 mAb at apparent molecular weights corresponding to those of the alpha and beta chains of the human C5 protein. The two 5G1.1 immunoreactive bands seen on this Western blot were subsequently found to result from the binding of the 5G1.1 antibody to the 115 kDa C5 alpha chain and to a large fragment of the alpha chain that had the same apparent molecular weight (approximately 75 kDa) as the beta chain of C5 and was present in the C5 preparations used for the experiment.

Assays were performed to determine the relative activity of the N19/8 mAb discussed in Examples 4 and 5 with the 5G1.1 mAb in functional hemolytic assays and to assess whether these mAbs blocked the cleavage of C5 to yield C5a. To this end, the N19/8 and 5G1.1 mAbs were directly compared in human complement hemolytic and C5a release assays.

Hemolytic assays performed in the presence of 20% v/v human serum revealed that the 5G1.1 mAb effectively blocked serum hemolytic activity at a final concentration of 6.25 μg/ml (0.5/1 molar ratio of 5G1.1/C5) whereas the N19/8 mAb blocked at a higher concentration of 25.0 μg/ml (2.0/1 molar ratio of N19/8/C5). When the supernatants from these assays were tested for the presence of C5a, the 5G1.1 mAb was found to have effectively inhibited C5a generation at doses identical to those required for the blockade of C5b-9 mediated hemolytic activity.

In contrast, the N19/8 mAb was 10 fold less effective in blocking the release of C5a in these assays when compared to the 5G1.1 mAb. Furthermore, the ability of the N19/8 mAb to block complement mediated hemolysis was not equivalent to its capacity to block C5a generation in that a dose of 25 μg/ml of N19/8 completely blocked hemolysis while only reducing C5a generation by 37%.

Hybridoma 5G1.1 was deposited with the American Type Culture Collection, 10801 University Boulevard, Manassas, Va. 20110-2209, United States of America, on Apr. 27, 1994, and has been assigned the designation HB-11625. This deposit were made under the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purposes of Patent Procedure (1977).

Example 8

Determination of the Affinity Constants (K_D) for the Anti-human C5 Monoclonal Antibodies 5G1.1 and N19/8

The procedure utilized to determine the dissociation constant (K_D) of antibody-antigen equilibria in solution was that described by Friguet et al., J. Immunol. Meth. 1985, 77:305–319. This method was used to determine the K_D for the anti-human C5 monoclonal antibodies N19/8 and 5G1.1. The monoclonal antibodies were incubated with the antigen (C5) in solution until the equilibrium was reached. The proportion of antibody that remains unbound (free) at equilibrium was measured using a conventional Enzyme Linked Immunosorbant Assay (ELISA). The experimental values of K_D obtained by this method have been shown to be equivalent to those obtained by other methods (immunoprecipitation of the radiolabeled antigen and fluorescence transfer). This method offers the advantage of dealing with unmodified antigen.

FIGS. 8 and 9 show the Scatchard plots of the binding of the anti-human C5 monoclonal antibodies 5G1.1 and N19/8 to human C5 as measured by ELISA. In each graph (v) represents the fraction of bound antibody and (a) represents the concentration of free antigen at equilibrium. The calculated K_D for the 5G1.1 mAb was 30 pM while the calculated K_D for the N19/8 mAb was 43 pM. These results indicate that the K_D for the 5G1.1 and N19/8 mab's are similar, and therefore the functional disparity between the two antibodies cannot be explained simply by the differences in affinity for the C5 antigen.

Example 9

Effect of 5G1.1 mAb on Complement Activation During CPB

Experiments involving recirculation of human blood in an CPB circuit, as described above in Examples 4 and 5, were carried out using three doses of the 5G1.1 mAb (15 mg, 7.5 mg, 3.75 mg) as well as controls in the absence of the 5G1.1 mAb. In five such control experiments performed in this series, C3a FIG. 10) and sC5b-9 (FIG. 11) levels increased during the first 30 min and continued to rise throughout the entire experiment. Addition of the 5G1.1 mAb to the CPB circuit had no effect on the generation of C3a in these experiments.

Conversely, addition of the two highest doses (15 mg and 7.5 mg) of the 5G1.1 mab completely blocked the generation of sC5b-9 in these experiments while the lowest dose (3.75 mg) only partially blocked sC5b-9 generation. Hemolytic assays performed on serum samples drawn throughout the time course of these experiments revealed that total serum complement activity was not affected in control experiments (FIG. 12). In contrast, the highest dose of the 5G1.1 mAb (15 mg) completely blocked complement hemolytic activity, while the two lower doses (7.5 mg and 3.75 mg), failed to block hemolytic activity.

These results show that the 7.5 mg dose effectively blocked C5b-9 generation in the CPB circuit but failed to block C5b-9-mediated hemolytic activity, suggesting that hemolytic assays alone may not accurately reflect the complement activation that occurs during CPB. These results further indicate that the 5G1.1 mAb can completely block complement activation in human blood, as measured by either criterion, at a dosage of 15 mg/500 ml, a dose that is approximately equivalent to a dose of 150 mg for a 70 kg patient.

Example 10

Cloning of Anti-C5 Recombinant Anti-KSSKC Variable Region Genes Amino Acid Sequencing

To determine the N-terminal amino acid sequence of the 5G1.1 mAb, a 12% acrylamide gel (37.5:1 acrylamide/N, N'-methylene-bisacrylamide) was prepared and pre-electrophoresed for 45 minutes at 10 mA using 1× pre-electrophoresis buffer (123 mM bis-Tris, pH 6.6, with the cathode buffer reservoir supplemented with 1 mM reduced glutathione). The following day, the pre-electrophoresis buffer in the cathode reservoir was replaced with cathode reservoir buffer (44 mM N-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid, 113 mM bis-Tris, 0.1% (w/v) sodium dodecyl sulfate (SDS), 0.067% (w/v) thioglycolic acid) and the pre-electrophoresis buffer in the anode reservoir was replaced with anode reservoir buffer (63 mM bis-Tris, pH 5.9).

75 μg 5G1.1 monoclonal antibody was added to Laemmli sample buffer (30 mM Tris-HCl pH 6.8, 3% (w/v) SDS, 10 mM EDTA, 0.02% (w/v) bromophenol blue, 5% (v/v) glycerol, 2.5% (v/v) beta-mercaptoethanol) and electrophoresed at 10 mA until the bromophenol blue tracking dye reached the bottom of the gel. The protein was transferred to a PROBLOTT membrane (Applied Biosystems, Foster City, Calif.) using 1× transfer buffer (10 mM cyclohexylaminopropane sulfonic acid, 0.05% (w/v) dithiothreitol, 15% (v/v) methanol) at 50 V for one hour.

Protein bands were localized by staining with 0.2% Ponceau S (in 3% trichloroacetic acid, 3% sulfosalicylic acid) followed by destaining with water. Bands were excised and subjected to amino acid sequence analysis using Edman chemistry performed on a pulsed liquid protein sequencer (ABI model 477A), with the PTH amino acids thereby obtained being analyzed with an on-line microbore HPLC system (ABI model 120A).

To deblock the amino terminus of the 5G1.1 heavy chain, 10 mg 5G1.1 monoclonal antibody was exchanged into reducing buffer (5 M guanidine-HCl, 50 mM Tris-HCl, 10 mM dithiothreitol, pH 8.5) using a PD-10 column (Pharmacia, Piscataway, N.J.). After a one hour incubation at room temperature, 50 mM iodoacetamide was added and the incubation allowed to continue for 30 minutes. The carbamidomethylated light and heavy chains thus obtained were separated by size exclusion chromatography on a SUPEROSE 12 (Pharmacia) column equilibrated with 5 M guanidine-HCl, 50 mM Tris-HCl pH 8.5. The carbamidomethylated heavy chain was exchanged into 50 mM sodium phosphate, pH 7.0 using a PD-10 column, subjected to digestion with pyroglutamate aminopeptidase (PanVera, Madison, Wis.; 0.5 mU per nmol of heavy chain protein), and sequenced as described above.

For determination of internal amino acid sequence, the carbamidomethylated 5G1.1 light chain was exchanged into 2 M urea, 25 mM Tris-HCl, 1 mM EDTA, pH 8.0 and incubated with endoproteinase Lys-C (Promega, Madison, Wis.; protease:protein ratio of 1:40) at 37° C. overnight. The digested material was run on a C18 reversed phase HPLC column (Beckman Instruments, Fullerton, Calif.) and eluted using a linear 0–50% acetonitrile gradient in 0.1% trifluoroacetic acid. Peaks were subjected to amino acid sequence analysis as described above.

PCR Cloning

Cloning of the 5G1.1 variable heavy region was performed using a set of commercially available primers (Mouse Ig-PRIMER SET, catalogue number 69831-1, Novagen, Madison, Wis.). Total RNA was isolated from 5G1.1 hybridoma cells using the acid/guanidinium thiocyanate technique (Chomczynski and Sacchi, Anal. Biochem. 1987, 162:156–159). For first strand cDNA synthesis, ten micrograms total RNA were denatured at 65° C. for 5 min., chilled on ice, and added to a 100 μl reaction containing 10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM dithiothreitol, 250 μM each dNTP, 20 units AMV reverse transcriptase (Seikagaku America, Rockville, Md.), and 10 pmole of the appropriate 3' primer (as described in the Ig-PRIMER SET kit protocol). After incubation at 37° C. for one hour, five microliters of the cDNA synthesis reaction were added to a 100 microliter PCR reaction containing: 10 mM Tris-HCl pH 9.0 at 25° C., 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% (w/v) gelatin, 1.0% (v/v) Triton X-100, 200 μM each dNTP, 2.5 U AMPLITAQ DNA polymerase (Perkin-Elmer-Cetus, Norwalk, Conn.) and 25 pmoles of the appropriate 5' and 3' primers (as described in the Ig-PRIMER SET kit protocol). The reaction conditions were 1 minute at 95° C., 1 minute at 42° C., and 1 minute at 72° C. for 30 cycles, followed by a final extension at 72° C. for 10 minutes.

PCR products having the expected size (approximately 450 bp) were cloned into the vector PCRII (Invitrogen, San Diego, Calif.) using a T/A cloning kit (Invitrogen). DNA sequence analysis of cloned DNA fragments was performed by the dideoxy chain-termination method using double-stranded plasmid DNA as a template. A unique heavy chain variable region was isolated by this procedure, with the resulting plasmid designated p5G1.1 VH 2-1-3. Several clones obtained from independent replicate PCR reactions were sequenced to detect any mutations introduced during the PCR amplification of this variable region.

To clone the 5G1.1 light chain variable region, PCR primers were designed by using the UWGCG program TFASTA (University of Wisconsin, Madison, Wis.) to search the GenBank rodent subdirectory with the 19mer query amino acid sequence Ile Gln Met Thr Gln Ser Pro Ala Ser Leu Ser Ala Ser Val Gly Glu Thr Val Thr, that was obtained by amino acid sequencing as described above. An exact match to this sequence was located in the murine germline gene encoding the v-kappa k2 variable region (Seidman et al. Proc. Natl. Acad. Sci. USA 1978 75:3881–3885). The DNA sequence of this germline gene was used to design the oligonucleotide UDEC690 (SEQ ID NO:5) for use as a variable region 5'-primer. A murine kappa gene constant region primer, UDEC395 (SEQ ID NO:6) was also synthesized and used in this reaction. Cloning of the 5G1.1 variable light region was performed using the UDEC690 variable region 5'-primer and the UDEC395 murine kappa gene constant region primer.

PolyA mRNA was isolated from hybridoma 5G1.1. The acid/guanidinium thiocyanate procedure (Chomczynski and Sacchi, supra) was used to isolate total RNA, and was followed by oligo(dT)-cellulose chromatography of 1 mg of total RNA. For first strand cDNA synthesis, one microliter of the 25 microliters of oligo(dT)-cellulose eluate (containing approximately 2 micrograms of purified 5G1.1 mRNA) was denatured at 65° C. for 5 min., chilled on ice, and incubated in extension buffer (10 mM Tris pH 8.3, 50 mM KCl, 1 mM dithithreitol, 240 μM each dNTP) containing 100 nM UDEC395 (SEQ ID NO:6) and 25 units AMV reverse transcriptase (Seikagaku America, Rockville, Md.) at 42° C. for one hour. Five microliters of the completed first strand reaction was subjected to PCR amplification using amplification buffer supplemented with 2.5 units AMPLITAQ DNA polymerase (Perkin Elmer, Foster City, Calif.) and 500 nM each of primer UDEC690 (SEQ ID NO:5) and UDEC395 (SEQ ID NO:6). Amplification was performed using 30 cycles each consisting of 1 minute at 95° C., 1 minute at 52° C., and 1 minute at 72° C., followed by a single ten minute incubation at 72° C.

The resulting PCR product was purified using GENECLEAN according to the manufacturer's directions (Bio 101, La Jolla, Calif.), digested with Sse8387 I and Hind III, gel purified, and ligated into the vector Bluescript II SK$^+$ (Stratagene, La Jolla, Calif.). Ligated plasmids were transformed into the bacterial strain DH10B by electroporation.

Plasmid DNA was purified from cultures of transformed bacteria by conventional methods including column chromatography using a QUIAGEN-TIP-500 column according to the manufacturer's directions (Quiagen, Chatsworth, Calif.) and sequenced by the Sanger dideoxy chain termination method using SEQUENASE enzyme (U.S. Biochemical, Cleveland, Ohio). Clones obtained from a second independent PCR reaction verified that no mutations were introduced during the amplification process. The resulting plasmid containing the cloned variable region was designated SK (+) 690/395. This light chain encoding insert in this plasmid coded for both the N-terminal and internal light chain sequences determined by amino acid sequencing of 5G1.1, as described above.

Example 11

Construction and Expression of Recombinant mAbs

Recombinant DNA constructions encoding the recombinant mAbs comprising the 5G1.1 CDRs are prepared by conventional recombinant DNA methods including restriction fragment subcloning and overlapping PCR procedures. The resulting recombinant mAb-encoding DNAs include:

(1) one encoding a non-humanized (murine) scFv designated 5G1.1M1scFv (SEQ ID NO:7), wherein CDR L1 is amino acid residues 28–34 of SEQ ID NO:7, CDR L2 is amino acid residues 52–54 of SEQ ID NO:7,

CDR L3 is amino acid residues 93–98 of SEQ ID NO:7, CDR H1 is amino acid residues 156–159 of SEQ ID NO:7, CDR H2 is amino acid residues 179–183 of SEQ ID NO:7, and CDR H3 is amino acid residues 226–236 of SEQ ID NO:7;

(2) one encoding a humanized (CDR grafted) scFv designated 5G1.1 scFv CB (SEQ ID NO:8), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:8, CDR L2 is amino acid residues 52–58 of SEQ ID NO:8, CDR L3 is amino acid residues 91–99 of SEQ ID NO:8, CDR H1 is amino acid residues 152–161 of SEQ ID NO:8, CDR H2 is amino acid residues 176–192 of SEQ ID NO:8, H3 is amino acid residues 225–237 of SEQ ID NO:8;

(3) one encoding a chimeric light chain (which can form the light chain portion of an Fab) designated 5G1.1M1 VL HuK (SEQ ID NO:9);

(4) one encoding a chimeric Fd (the heavy chain portion of an Fab) designated 5G1.1M1 VH HuG1 (SEQ ID NO:10);

(5) one encoding a humanized (CDR grafted and framework sequence altered) Fd designated 5G1.1 VH+IGHRL (SEQ ID NO:11), wherein CDR H1 is amino acid residues 26–35 of SEQ ID NO:11, CDR H2 is amino acid residues 50–60 of SEQ ID NO:11, and CDR H3 is amino acid residues 99–111 of SEQ ID NO:11;

(6) one encoding a humanized (CDR grafted, not framework altered) Fd designated 5G1.1 VH+IGHRLC (SEQ ID NO:12), CDR H1 is amino acid residues 26–35 of SEQ ID NO:12, CDR H2 is amino acid residues 50–66 of SEQ ID NO:12, and CDR H3 is amino acid residues 99–111 of SEQ ID NO:12;

(7) one encoding a humanized (CDR grafted and framework sequence altered) light chain designated 5G1.1 VL+KLV56 (SEQ ID NO:13), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:13, CDR L2 is amino acid residues 52–58 of SEQ ID NO:13, and CDR L3 is amino acid residues 91–99 of SEQ ID NO:13;

(8) one encoding a humanized (CDR grafted, not framework altered) light chain designated 5G1.1 VL+KLV56B (SEQ ID NO:14), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:14, CDR L2 is amino acid residues 52–58 of SEQ ID NO:14, and CDR L3 is amino acid residues 91–99 of SEQ ID NO:14;

(9) one encoding a humanized (CDR grafted, not framework altered) light chain designated 5G1.1 VL+012 (SEQ ID NO:15), wherein CDR L1 is amino acid residues 24–34 of SEQ ID NO:15, CDR L2 is amino acid residues 50–56 of SEQ ID NO:15, and CDR L3 is amino acid residues 89–97 of SEQ ID NO:15; and

(10) one encoding a humanized (CDR grafted, not framework altered) Fd designated 5G1.1 VH+IGHRLD (SEQ ID NO:16), wherein CDR H1 is amino acid residues 26–35 of SEQ ID NO:16, CDR H2 is amino acid residues 50–60 of SEQ ID NO:16, and CDR H3 is amino acid residues 99–111 of SEQ ID NO:16.

(11) one encoding a humanized (CDR grafted) scFv designated 5G1.1 scFv DO12 (SEQ ID NO:17), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:17, CDR L2 is amino acid residues 52–58 of SEQ ID NO:17, CDR L3 is amino acid residues 91–99 of SEQ ID NO:17, CDR H1 is amino acid residues 152–161 of SEQ ID NO:17, CDR H2 is amino acid residues 176–186 of SEQ ID NO:17, and CDR H3 is amino acid residues 225–237 of SEQ ID NO:17;

(12) one encoding a humanized (CDR grafted and framework sequence altered) scFv designated 5G1.1 scFv CO12 (SEQ ID NO:20), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:20, CDR L2 is amino acid residues 52–58 of SEQ ID NO:20, CDR L3 is amino acid residues 91–99 of SEQ ID NO:20, CDR H1 is amino acid residues 152–161 of SEQ ID NO:20, CDR H2 is amino acid residues 176–192 of SEQ ID NO:20, H3 is amino acid residues 225–237 of SEQ ID NO:20;

(13) one encoding a humanized (CDR grafted) scFv designated 5G1.1 scFv DO12B (SEQ ID NO:21), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:21, CDR L2 is amino acid residues 52–58 of SEQ ID NO:21, CDR L3 is amino acid residues 91–99 of SEQ ID NO:21, CDR H1 is amino acid residues 152–161 of SEQ ID NO:21, CDR H2 is amino acid residues 176–192 of SEQ ID NO:21, H3 is amino acid residues 225–237 of SEQ ID NO:21;

(14) one encoding a humanized (CDR grafted) scFv designated 5G1.1 scFv DO12C (SEQ ID NO:22), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:22, CDR L2 is amino acid residues 52–58 of SEQ ID NO:22, CDR L3 is amino acid residues 91–99 of SEQ ID NO:22, CDR H1 is amino acid residues 152–161 of SEQ ID NO:22, CDR H2 is amino acid residues 176–192 of SEQ ID NO:22, H3 is amino acid residues 225–237 of SEQ ID NO:22;

(15) one encoding a humanized (CDR grafted) scFv designated 5G1.1 scFv DO12D (SEQ ID NO:23), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:23, CDR L2 is amino acid residues 52–58 of SEQ ID NO:23, CDR L3 is amino acid residues 91–99 of SEQ ID NO:23, CDR H1 is amino acid residues 152–161 of SEQ ID NO:23, CDR H2 is amino acid residues 176–192 of SEQ ID NO:23, H3 is amino acid residues 225–237 of SEQ ID NO:23;

(16) one encoding a humanized (CDR grafted and framework sequence altered) scFv designated 5G1.1 scFv CO13 (SEQ ID NO:24), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:24, CDR L2 is amino acid residues 52–58 of SEQ ID NO:24, CDR L3 is amino acid residues 91–99 of SEQ ID NO:24, CDR H1 is amino acid residues 152–161 of SEQ ID NO:24, CDR H2 is amino acid residues 176–192 of SEQ ID NO:24, H3 is amino acid residues 225–237 of SEQ ID NO:24;

(17) one encoding a humanized (CDR grafted and framework sequence altered) scFv designated 5G1.1 scFv CO14 (SEQ ID NO:25), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:25, CDR L2 is amino acid residues 52–58 of SEQ ID NO:25, CDR L3 is amino acid residues 91–99 of SEQ ID NO:25, CDR H1 is amino acid residues 152–161 of SEQ ID NO:25, CDR H2 is amino acid residues 176–192 of SEQ ID NO:25, H3 is amino acid residues 225–237 of SEQ ID NO:25;

(18) one encoding a humanized (CDR grafted and framework sequence altered) scFv designated 5G1.1 scFv CO15 (SEQ ID NO:26), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:26, CDR L2 is amino acid residues 52–58 of SEQ ID NO:26, CDR L3 is amino acid residues 91–99 of SEQ ID NO:26, CDR H1

is amino acid residues 152–161 of SEQ ID NO:26, CDR H2 is amino acid residues 176–192 of SEQ ID NO:26, H3 is amino acid residues 225–237 of SEQ ID NO:26;

In accordance with the invention, one each of the various L1, L2 and L3 CDRs discussed in (1) to (18) above may be combined with any of the other light chain CDRs so as to make a set of 3 light chain CDRs comprising one L1, one L2, and one L3 CDR, as part of a recombinant antibody or synthetic peptide antibody (i.e., a synthetic peptide with the sequence of a recombinant peptide of the invention). Furthermore, the framework regions (i.e., the regions not included in the CDRs as described for each) of each of (1) to (18) above may be interchanged with homologous framework regions of the other recombinant antibody molecules of (1) to (18) to produce other antibodies of the invention.

In accordance with the invention, one each of the various H1, H2 and H3 CDRs discussed in (1) to (18) above may be combined with any of the other light chain CDRs so as to make a set of 3 light chain CDRs comprising one H1, one H2, and one H3 CDR, as part of a recombinant antibody or synthetic peptide antibody (i.e., a synthetic peptide with the sequence of a recombinant peptide of the invention).

In accordance with the invention, matched pairs of the variable regions (e.g., a VL and a VH region) of the various antibody molecules, Fds, and light chains described above may be combined with constant region domains by recombinant DNA or other methods known in the art to form full length antibodies of the invention. Particularly preferred constant regions for this purpose are IgG constant regions, which may be unaltered, or constructed of a mixture of constant domains from IgGs of various subtypes, e.g., IgG1 and IgG 4.

Matched pairs of the Fd and light chain encoding DNAs described immediately above—i.e. (3) and (4), (5) and (7), (6) and (8), and (6) and (9)—were subcloned together into the APEX-3P vector, essentially as described below in Example 15 for N19/8. The scFv constructs of (1) and (2) were subcloned into pET Trc SO5/NI using conventional techniques.

Plasmids so obtained were introduced by into the bacterial strain ME2 (pET plasmids) by conventional electroporation, or into human 293 EBNA cells (APEX plasmids) by lipofection using 2–3 microliters of TRANSFECTAM reagent (Promega, Madison, Wis.) per microgram of DNA according to the manufacturer's directions. Bacterial strains ME1 and ME2 are derivatives of *Escherichia coli* strain W3110 (ATCC designation 27325) prepared as follows.

Preparation of W3110 Derivatives ME1 and ME2

The non-humanized, non-chimeric murine 5G1.1-scFv "m5G1.1-scFv"—made up of light chain (3) and Fd (4)—was expressed in a derivative of *E. coli* K12 strain W3110. This derivative was prepared by inactivating an uncharacterized gene to provide protection against infections by a lytic bacteriophage. *E. coli* strain W3110 is a particularly preferred strain because it is fully characterized and is commonly used for recombinant DNA product fermentations.

A single colony of *E. coli* strain W3110 was grown overnight in L medium at 30° C. The cells were collected by centrifugation and resuspended in 10 mM $MgSO_4$. A total of 0.1 ml of the culture was added to 2.5 ml 0.7% L soft agar at 45° C. and quickly poured on an L plate. Fifty microliter aliquots of a plaque purified phage lysate, undiluted, diluted 10^{-2} and diluted 10^{-4}, were spotted onto the agar surface. Phage lysates had previously been filtered through 0.45 μm membranes and stored in sterile tubes with a drop of chloroform at 4° C. The spots were allowed to dry on the soft agar surface and incubated overnight at 37° C.

The next day L plates were spread with 10^9 phage PFU and allowed to dry. Using a sterile, flat toothpick, cells from isolated colonies growing in the zones of phage lysis on the spot plates were streaked for single colonies on the plates spread with 10^9 phage PFU and incubated overnight at 37° C. Single colonies were rechecked for phage resistance by cross-streaking after single colony purification. The cross streak test for phage sensitivity was performed as follows. Fifty μl of phage (10^8 pfu/ml) was spread in a vertical line in the left hand portion of the plate using a Pasteur pipette. Additional phage were tested parallel to the first and to the right. The plate was allowed to dry, and strains to be checked for sensitivity or resistance were spread perpendicular to and across the lines of all phages in a single swath from the left to the right. Resistant strains grow in the area of the phage streaks while sensitive strains lyse.

The phage resistant mutant strain ME1 was tested for phage production after overnight growth in L medium and treatment with the DNA damaging agent, mitomycin C. The strain failed to produce viable phage utilizing a standard plaque assay and *E. coli* W3110 as the phage sensitive indicator strain. These results suggest that strain ME1 does not harbor a resident prophage.

Strain ME2 was constructed by site specific integration of the lambdaDE3 prophage (Studier et al. 1990, Meth. Enzymol. 185:60–89) into the ME1 chromosome. Expression of the T7 RNA polymerase, directed by the prophage, allows expression of target genes cloned into pET vectors (Studier et al., supra) under the control of the T7 promoter in the lysogenized host. Lysogenization was accomplished in a three way infection with lambdaDE3, the lambda helper phage, lambdaB10 and the selection phage, lambdaB482 (Studier et al., supra).

lambdaDE3 (imm21) was constructed by Studier and colleagues (1990, Meth. Enzymol. 185:60–89) by inserting the T7 RNA polymerase gene behind the *E. coli* lacUV5 promoter into the BamHI cloning site of lambdaD69 (imm21). Since cloning into the BamHI site of lambdaD69 interrupts the integrase gene, lambdaDE3 cannot integrate or excise from the chromosome by itself. The helper phage lambdaB10 provides the integrase function that lambdaDE3 lacks but cannot form a lysogen by itself. The selection phage, lambdaB482, lyses any lambdaDE3 host range mutants that otherwise would be among the surviving cells, but it can neither integrate into susceptible cells nor lyse lambdaDE3 lysogens since it has the same immunity region as lambdaDE3 (imm21).

Lysoaenization Protocol

Strain ME1 was grown in L medium supplemented with 0.2% maltose and 10 MM $MgSO_4$ at 37° C. to a density of approximately 10^8 cells/ml. One μl of ME1 cells were incubated with 2×10^8 plaque forming units (pfu) of lambdaDE3 and 10^8 pfu of lambdaB10 and lambdaB482. The host/phage mixture was incubated at 37° C. for 20 min to allow phage adsorption to ME1 cells. Several dilutions of the cell/phage suspension were spread on L plates to produce plates containing approximately 30–200 candidate lysogens as isolated colonies. The plates were inverted and incubated at 37° C. overnight. Several isolated colonies were screened for the acquisition of the lambdaDE3 prophage as described below.

Verification of lambdaDE3 Lysogens

lambdaDE3 lysogen candidates were tested for their ability to support the growth of the T7 phage 4107, a T7 phage deletion mutant that is completely defective unless active T7

RNA polymerase is provided in trans. Only lambdaDE3 lysogens will support the normal growth of the phage in the presence of IPTG (isopropyl-beta-thiogalactopyranoside). The T7 phage produces very large plaques on lambdaDE3 lysogens in the presence of IPTG, while very small plaques are observed in the absence of inducer. The size of the plaque in the absence of IPTG is an indication of the basal level of T7 RNA polymerase expression in the lysogen. Putative lambdaDE3 lysogens were grown in L broth supplemented with 0.2 % maltose and 10 mM $MgSO_4$ at 37° C. to a cell density of approximately 10^8 cells/ml. A total of 0.5 ml of cells was centrifuged and the pellet was resuspended in 0.2 ml of a T7 phage lysate containing 2×10^4 pfu. The phage was allowed to adsorb for 30 min at 37° C. One-half of suspension (0.1 ml) was added to 3.0 ml of molten top agarose at 47° C. and poured onto L plates. The remaining aliquot of cell/phage suspension was poured onto an L plate supplemented with 0.4 mM IPTG to check for induction of T7 RNA polymerase. The plates were inverted and incubated at 37° C. overnight.

Strains were also tested for the presence of the lambdaDE3 lysogen by demonstrating that each strain was resistant to infection by the phage lambdaB482, which is in the same immunity group (imm21), by the cross streak method described above. A lysogen was chosen with a low basal expression level for protein production from pET vectors. The resulting strain, designated ME2, is phage resistant and overexpresses T7 RNA polymerase in the presence of IPTG.

Purification of Humanized 5G1.1-scFv from *E. coli*

The humanized 5G1.1-scFv (h5G1.1-scFv) cDNA construct was cloned into the bacterial expression plasmid pET Trc SO5/NI (SEQ ID NO:18) and transformed into *E. coli* strain ME1. The resulting strain expressing h5G1.1 scFv was grown at 37° C. in 2 liter Applikon glass vessel fermentors containing Terrific Broth (1.2 % (w/v) bacto-tryptone, 2.4% (w/v) bacto-yeast extract, 0.4% (v/v) glycerol, 90 mM KPO_4, pH 7.0) supplemented with 100 μg/ml ampicillin. The production of recombinant scFv was induced by the addition of 1 mM IPTG when the $O.D._{550}$ of the culture reached 10. After an additional 3 h incubation at 37° C., the cells were harvested by centrifugation and the cell pellets stored at −80° C.

Cells were resuspended in 1 mM EDTA, pH 5.0 at 10 ml per gram weight and lysed by a single pass through a microfluidizer (Model M110T, Microfluidics Corp., Newton, Mass.). After centrifugation at 17,500×g for 15 min, the resulting inclusion body pellet was washed by resuspension in 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.15% (w/v) deoxycholate at 10 ml per gram inclusion body using a Tekmar POLYTRON. The inclusion bodies were again pelleted by centrifugation at 17,500×g for 15 min and resuspended in 20 mM Tris-HCl pH 9.0, 8 M urea at 10 ml per g. After stirring for 1 h, the sample was centrifuged at 14,000×g for 30 min to pellet remaining insoluble material.

The extract supernatant was diluted 10-fold with 20 mM Tris-HCL pH 9.0, 7 M urea, 50 μM cupric sulfate and allowed to stir for at least 16 hours at 4° C. to refold the scFv. After addition of BIOCRYL BPA-1000 (TosoHaas, Montgomeryville, Pa.) as a flocculating agent at 3 μl per ml, the sample was centrifuged at 15,000×g for 10 minutes to remove insoluble material. The refolding mixture was exchanged into 20 mM Tris, pH 9.0, 1 mM EDTA by diafiltration and concentrated by ultrafiltration using a stirred cell fitted with a YM10 membrane (Amicon, Beverly, Mass.).

In subsequent experiments, other refolding conditions were tested. Thawed bacterial cells were resuspended with a POLYTRON homogenizer in 1 mM EDTA at 2.5 mL per gram of cells, passed through the MICROFLUIDIZER at 18,000 psi, and centrifuged at 10,000 RPM for 15 min in a Beckman JA-10 rotor, the resulting pellet was washed by resuspension in 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.15% (w/v) deoxycholate at 10 ml per gram inclusion body using a Tekmar POLYTRON. The inclusion bodies were again pelleted by centrifugation. The pellet from this centrifugation was resuspended with a POLYTRON homogenizer in 8M urea, 20 mM Tris pH9 at 10 mL per gram of pellet. After stirring for 1 hour at 4 degrees C., the resuspended pellet was diluted with 9 volumes of 7M urea, 20 mM Tris pH9. Cupric sulfate was then added to various final concentrations (0, 5, 10, 20, 25, 30, 40, 50, 100, 150, and 200 μM) before incubation overnight at 4 degrees C. with stirring. The use of 5 μM copper was found to give the highest levels of refolding of the humanized 5G1.1-scFv as assessed by analytical HPLC.

In the initial experiments, the properly refolded scFv was then separated from aggregated material and contaminating proteins by anion exchange chromatography using Q SEPHAROSE FAST FLOW (Pharmacia, Piscataway, N.J.). Bound scFv was eluted with 20 mM Tris-HCL pH 9.0, 1 mM EDTA containing a linear NaCl gradient (0 to 0.5 M). The fractions containing the scFv were combined, concentrated by ultrafiltration using a stirred cell fitted with a YM10 membrane, and applied to a SEPHACRYL S200 HR 26/100 gel filtration column (Pharmacia) equilibrated in 20 mM Tris-HCL pH 9.0, 1 mM EDTA, 150 mM NaCl. Fractions containing the scFv were combined, exchanged into phosphate-buffered saline by diafiltration, concentrated by ultrafiltration, filtered through a 0.22 μm MILLEX-GV filter (Millipore, Bedford, Mass.), and stored at 4° C.

Subsequent experiments have indicated that cation exchange chromatography (e.g., using POROS HS resin—PerSeptive Biosystems, Cambridge, Mass.) should give better yields than the Q Sepharose Fast Flow anion exchange chromatography step described in the preceding paragraph. In addition, it would be preferable to carry out the final gel filtration chromatography in a buffer that is more pharmaceutically acceptable than the Tris buffer described. A buffer such as PBS would be preferred if it does not interfere with the efficacy of the gel filtration chromatographic separation. This would reduce any trace amounts of Tris remaining in the preparation after diafiltration, and might eliminate the need for the diafiltration step.

Purification of m5G1.1-scFv from *E. coli*

Frozen bacterial cell paste was thawed and resuspended in 2.5 ml of 1 MM EDTA (pH 5) per gram of cell paste. This suspension of cells was lysed by passage through a MICROFLUIDIZER (Microfluidics) with the interaction chamber in line and a backpressure of approximately 18000 psi. The cell lysate was then centrifuged at 10,000 rpm in a JA-10 centrifuge rotor at 4° C. for 15 min. The supernatant was decanted and discarded.

The pellet was resuspended in 10 ml of 20 mM Tris, pH 8.0, 100 mM NaCl, 0.15% sodium deoxycholate per gram of pellet. This suspension was centrifuged as above for 10 min. Again the supernatant was decanted and discarded. This detergent washed pellet was then resuspended in 10 ml of 8 M urea, 20 mM Tris-HCl, pH 9 (1 mM EDTA may also be added to this buffer, but has the effect of increasing the time required to achieve a particular level of refolding). The suspension was stirred at 4° C. for 1 hr. and was then diluted 10 fold with 7 M urea, 20 mM Tris-HCl, pH 9 and stirred at 4° C. $CUSO_4$ was then added to a final concentration of 50 μM and stirring was continued overnight at 4° C.

The majority of contaminating proteins (including incorrectly folded versions of m5G1.1 scFv) were then removed by precipitation by diluting (with stirring) the refolded sample five fold with buffer such that the final concentrations after dilution were 1.4 M urea, 25 mM NaCl, 1 mM EDTA, and 20 mM sodium acetate at 4° C. The pH of the dilution buffer when prepared at room temperature was pH 5.0. Prior to dilution the pH of the dilution buffer is determined at 4° C. After the dilution the pH of the sample was greater than pH 5.5. The pH of the sample was then adjusted with 6. N HCl to the initial pH 5.0 of the buffer at 4° C. The solution immediately became cloudy and it was left stirring at 4–8° C. for 0.5 to 24 hours.

The precipitate was removed by filtering the sample through a 300 kDa cut-off ultrafiltration membrane (Millipore Corporation, Bedford, Mass.). The permeate was collected and concentrated 5 fold using a 10 kDa cutoff ultrafiltration membrane (Millipore). This concentrated retentate was then diluted 2 fold with 20 mM sodium acetate, 1 mM EDTA, pH 5.0 in order to lower the NaCl concentration to 12.5 mM.

The diluted retentate was then loaded at 4° C. onto a SP SEPHAROSE FF column (Pharmacia) equilibrated in 0.7 M urea, 1 mM EDTA, 10 mM NaCl, 20 mM sodium acetate, pH 5.0, at a linear flowrate of 5 cm/min. Bed height was equal to or greater than 3.5 cm. Following loading the column was washed with 40 column volumes (CV) of equilibration buffer. The column was then washed with 20 CV of 20 mM sodium acetate, pH 5.0, 1 mM EDTA. The bound scFv was then eluted using 20 mM sodium citrate, pH 5.8, 1 mM EDTA. A single peak was collected in approximately 4 column volumes.

The SP SEPHAROSE eluate was then adjusted to 20 mM Tris-HCL by addition of 1 M Tris-HCL, pH 8. The pH of the sample was adjusted to 8.0 by addition of 1 N NaOH. This sample was loaded onto a Q SEPHAROSE FF column (Pharmacia) equilibrated in 20 mM Tris-HCL, pH 8.0, 1 mM EDTA at room temperature at a flowrate of 5 cm/min. The flow through fraction containing the scFv was collected.

The Q SEPHAROSE flow through fraction was then adjusted to 150 mM NaCl and concentrated to 10 mg of scFv per ml using a 10 kDa cutoff ultrafiltration membrane. This concentrated sample was then loaded onto a SEPHACRYL S200 column equilibrated in phosphate buffered saline, pH 7.4 and eluted at 0.4 cm/min. The fractions were analyzed by SDS-PAGE and silver staining. Peak fractions were combined after discarding the front and back shoulder fractions that contained the majority of contaminants.

Example 12

Functional Analysis of the m5G1.1 scFv

Titration of the m5G1.1 scFv in hemolytic assays revealed that the m5G1.1 scFv inhibited human complement-mediated lysis in a dose dependent fashion (FIG. 13). Direct comparison of the efficacy of the m5G1.1 scFv to the 5G1.1 mAb and Fab demonstrated that the m5G1.1 scFv completely blocked C5b-9-mediated hemolysis in 20% human serum at 0.15 μM while the 5G1.1 mAb and Fab blocked at 0.06–0.08 μM. Analysis of C5a generation in these assays revealed similar results in that the 5G1.1 scfv completely blocked C5a generation at 0.15 μM while the 5G1.1 mAb and Fab blocked at 0.06–0.08 μM (FIG. 14). Taken together these experiments indicated that unlike N19/8, which lost half of its effectiveness at blocking C5a generation upon being engineered as an scFv (SEQ ID NO:19), the 5G1.1 murine scFv retained the capacity to block the generation of both C5a and C5b-9.

Additionally, these data demonstrate that the m5G1.1 scFv retained similar activity to that of the parent molecule (the native murine 5G1.1 mAb) in that the molar concentration of 5G1.1 murine scFv required to completely block C5a and C5b-9 (0.15 μM) was within two-fold of that required for the 5G1.1 mAb and Fab (0.06–0.08 μM).

In order to determine whether the m5G1.1 scFv retained the capacity to block the activation of complement in the ex vivo model of cardiopulmonary bypass, 4.5 mg of the purified bacterially produced 5G1.1 murine scFv was added to the CPB circuit and complement activation was monitored. In control experiments, both C3a and C5b-9 levels increased throughout the time course of the experiment. In a single experiment, addition of 4.5 mg of the m5G1.1 scFv to the CPB circuit had no effect on the generation of C3a (FIG. 15). Conversely, complement hemolytic activity as well as the generation of sC5b-9 was completely blocked in this experiment (FIG. 16 and FIG. 17).

Example 13

Characterization of the Epitope Recognized by 5G1.1

Tryptic digestion: Twenty micrograms of purified human C5 (Advanced Technologies, San Diego, Calif.) was subjected to enzymatic digestion with 1 μg of TPCK-treated trypsin (Worthington Biochemical Corp., Freehold, N.J.). The digestion was allowed to continue for 3 minutes, after which time it was stopped by the addition of 20 μg soy bean trypsin inhibitor (Worthington). The reaction was then denatured and reduced by the addition of protein sample buffer and immediately boiled for 5 min. The digested fragments were size fractionated through a SDS-PAGE on a 12 % gel. The gel was then electroblotted in transfer buffer (20% (v/v) methanol, 25 mM Tris-base pH 8.0, and 192 mM glycine) to nitrocellulose (Bio-Rad Laboratories, Hercules, Calif.) and subjected to ECL western blot analysis using either 5G1.1 or a C5a specific monoclonal antibody (G25/2, obtained from Dr. Otto Götze, Department of Immunology, University of Göttingen, Germany).

The filters were incubated twice for 30 minutes each in blocking solution (500 mM NaCl, 5 mM Tris p-H 7.4, 10% (v/v) nonfat dry milk, and 0.2% (v/v) TWEEN-20). The filters were then changed to fresh blocking solution (20 ml) containing the primary antibody and incubated for 40 minutes on a rocking platform. The filters were rinsed briefly with washing solution (500 mM NaCl, 35 mM Tris pH7.4, 0.1% SDS, 1% NP40, and 0.5% deoxycholic acid) to remove any milk, and then fresh wash solution was added and incubated for two 20 minute intervals on an orbiting shaker. The filters were rinsed briefly with 10 to 20 mls of secondary antibody solution (500 mM NaCl, 5 mM Tris pH 7.4, 10% (v/v) Nonfat dry milk, 0.2% (v/v) TWEEN-20, and 1% NP-40) and then incubated with fresh secondary antibody solution containing a 1:2000 dilution of HRP conjugated goat anti-mouse for 20 minutes on a rocking platform. The filters were then washed as described above, incubated in ECL reagent (Amersham Corp., Arlington Heights, Ill.) for 1 minute and then exposed to ECL HYPERFILM (Amersham).

Acid Hydrolysis: Twenty micrograms of purified human C5 (Advanced Technologies) was subjected to hydrolysis in 1N acetic acid. The 20 μg of human C5 (1 μg/μl) was added to 20 μl of 2N acetic acid and incubated for 10 min at 100° C. The sample was denatured and reduced with protein sample buffer, also at 100° C., for 5 minutes. The acid was

neutralized by dropwise addition of a saturated tris base solution until the sample turned blue. The cleavage products were then size fractionated by SDS-PAGE and western blotted as described above. For N-terminal sequencing, the gel fractionated acid hydrolysate was transferred to PVDF membrane. N-terminal sequence was obtained by excising the 46 kDa acid hydrolysis fragment band from a PVDF membrane and subjecting it to amino acid sequence analysis as discussed above in Example 10.

Deglycosylation: Reduced and denatured acid hydrolyzed or tryptic fragments of human C5 were subjected to deglycosylation with N-Glycosidase F (Peptide-N-Glycosidase F, Boehringer Mannheim Corp., Indianapolis, Ind.) according to the manufacture's directions.

Results: Acid hydrolysis of human C5 yielded a fragment with an apparent molecular weight by SDS-PAGE of 46 kDa that was immunoreactive for both the anti-C5a mAb G25/2 and the anti-C5 alpha chain mAb 5G1.1. Western blots probed with both antibodies simultaneously, as well as silver stain SDS-PAGE analysis, confirmed the presence of a single 46 kDa fragment that was immunoreactive with both antibodies. The presence of a single immunoreactive fragment containing binding sites for both 5G1.1 and G25/2 strongly suggested that the 5G1.1 epitope was contained within approximately the first 46 kDa of the N-terminus of the alpha chain of C5.

As discussed above in the description of the complement system under the heading "Background Physiology & Pathology," a compound (e.g., an antibody) that binds to a site at or immediately adjacent to the C5a cleavage site would have the potential to act as a terminal complement inhibitor. The potential inhibitory activity of antibodies binding to this site led to the expectation that the C5 alpha chain-binding 5G1.1 antibody would bind to an epitope at or near the C5a cleavage site. The finding that 5G1.1 bound to the 46 kDa acid hydrolysis fragment of C5 lent support to this expectation.

Western blot analysis of the tryptic digestion products identified one proteolytic fragment migrating at approximately 27 kDa that was immunoreactive with 5G1.1. Likewise, one immunoreactive proteolytic fragment migrating at approximately 29 kDa was observed following western blot analysis with the anti-C5a mAb G25/2. Experiments in which a blot was simultaneously probed with both 5G1.1 and G25/2 demonstrated that each band was distinct and that their apparent differential mobility was not a gel anomaly. This was surprising, because the 5G1.1 mAb was thought likely to bind to the C5 convertase cleavage site. 5G1.1 was thus expected to be immunoreactive with any fragment of C5 of over 12 kDa that exhibited immunoreactivity with G25/2. Such a fragment would contain enough of the extreme amino terminus of the C5 alpha chain to bind specifically to the anti-C5a mAb, and enough beyond that to encompass a region including and extending beyond the C5 convertase cleavage site.

The immunoreactivity of G25/2 with the 29 kDa fragment indicated that that fragment contains the N-terminal region of the alpha chain of C5 that is cleaved off to yield C5a. Furthermore, because 5G1.1 was not immunoreactive with this band, the 5G1.1 epitope was not likely to be contained within approximately the first 29 kDa of the N-terminus of the alpha chain of C5, and therefore could not be located near the C5 convertase cleavage site.

These tryptic digestion and acid hydrolysis mapping data suggested that the 5G1.1 epitope was contained within a region starting about 29 kDa (including post-translational modifications) from the N-terminus of the alpha chain of C5 and continuing 17 kDa in a C-terminal direction, i.e., ending 46 kDa from the N-terminus, a surprising finding in view of the expectation, discussed above, that the antibody would bind at or immediately adjacent to the point at which C5a is cleaved off of the C5 alpha chain, i.e., at or immediately adjacent to amino acid residue 733 of SEQ ID NO:2.

Post-translational modifications can alter the mobility of proteins during SDS-PAGE electrophoresis. One such modification is the addition of carbohydrate via N-linked glycosylation. As discussed above under the heading "Background Physiology & Pathology", C5 is glycosylated, as is C5a. C5a is glycosylated at an asparagine residue corresponding to amino acid number 723 of the full length pro-C5 precursor of human C5 (SEQ ID NO:2).

Computer analysis of the human C5 alpha chain suggests potential N-linked glycosylation sites at positions corresponding to amino acid numbers 893, 1097, and 1612 of SEQ ID NO:2. In order to determine the contribution of carbohydrate to the electrophoretic mobility of both the tryptic and acid fragments, enzymatic deglycosylation of the fragments was performed and followed by western blot analysis. It was determined that each tryptic fragment lost approximately 3 kDa in apparent molecular weight while the acid fragment lost approximately 6 kDa.

This result was interpreted as indicating that the tryptic fragments were each glycosylated at a single site and that the 46 kDa acid fragment was glycosylated at two sites (one of which was the known glycosylation site in C5a referred to above). The diminished mobility observed following deglycosylation agrees with the computed prediction of a second N-linked glycosylation site within the first 233 amino acids of the C5 alpha chain.

N-terminal sequence analysis determined that the first four amino acids of the 46 kDa fragment generated by 1N acetic acid treatment was Thr Leu Gln Lys. This sequence is found only once in the full length human pro-C5 precursor molecule—at a position corresponding to amino acids 660 through 663 of SEQ ID NO:2. This four amino acid sequence also corresponds to the sequence of the amino-terminus of the alpha chain of human C5 and, thus to the amino-terminus of human C5a.

In order to more precisely map the binding site of 5G1.1, overlapping peptide analysis was performed. The sequence predicted to be contained within the 17 kDa section of human C5 described above (SEQ ID NO:2; amino acids 893 through 1019) together with an extension of 43 amino acids towards the N-terminus and 30 amino acids towards the C-terminus (a total of 200 amino acids) was synthesized as a series of 88 overlapping peptides by solid phase synthesis on polypropylene filters (Research Genetics Inc., Huntsville, Ala.).

The 43 and 30 amino acid extensions were added to allow for possible inaccuracies in the prediction of the span of this 17 kDa region. Such inaccuracies are likely due to the uncertainty of the specific extent of glycosylation of each of the various regions of C5a, as well as to the aberrant gel mobility that is commonly seen when highly charged polypeptides (such as the 5G46k fragment and the 5G27k fragment) are analyzed by SDS-PAGE. As discussed above in the Summary of the Invention, a 200 amino acid peptide corresponding to the region covered by these overlapping peptides is referred to herein as the "5G200aa" peptide.

Because of the expectation that the 5G1.1 antibody would bind at the C5a cleavage site, an additional set of 8 overlapping peptides was synthesized that spanned a 30 amino

acid section spanning the C5a cleavage site (amino acids 725 through 754 of SEQ ID NO:2). A peptide having the sequence of this 30 amino acid section is referred to herein as the "cleavage site peptide". A 325aa peptide spanning amino acid residues 725–1049 of SEQ ID NO:2 (this peptide spans the region covered by the cleavage site peptide and the 5G200aa peptide) is referred to herein as the "5G325aa" peptide.

These filters were probed with 5G1.1 as described above for ECL western blot analysis, and a set of 4 overlapping peptides spanning the region corresponding to amino acid residues 3–19 of the KSSKC peptide (SEQ ID NO:1) each gave a positive signal indicative of monoclonal antibody binding, while peptides corresponding to the C5a cleavage site did not bind to the 5G1.1 antibody.

Example 14

C3/C4 Binding Assay

C3 and C4 are both key components of classical C5 convertase, and C3 is also a key component of alternative C5 convertase. These C5 convertases are required for the conversion of C5 to active C5a and C5b. The ability to block C5 binding to C3 and C4 is thus a desirable property for an antibody to be used in treatment of complement mediated diseases in accordance with the present invention.

96 well microtiter plates were coated with 501 μ/well, 10 μg/ml of either purified human C3 or C4 (Quidel) for 1 hour at 37° C. The plates were then blocked with 200 μl/well of TBS containing 1% BSA for 1 hour at room temperature. After three washes in TBS 0.1% BSA, purified human C5 (Quidel, 20 μg/ml in TBS 1% BSA) was added to the plates in the presence (20 μg/ml) or absence of a 5G1.1 Fab (derived from 5G1.1 by conventional papain digestion) and allowed to incubate for 1 hour at 37° C. After three washes in TBS/0.1% BSA, a monoclonal antibody directed against the C5 beta chain (N19/8, 5 μg/ml) was added to the wells to detect C5 bound to either C3 or C4. After three final washes in TBS/0.1% BSA, the plate was developed using a horseradish peroxidase-conjugated secondary antibody and the appropriate substrate.

The results of these assays showed that the 5G1.1 mAb inhibited the binding of purified human C5 to either C3 or C4 by at least 60% to 90%. As used herein and in the claims, such a 60% to 90% reduction in C3 or C4 binding is a "substantial reduction" in C3 or C4 binding.

Example 15

Construction and Functional Analysis of N19/8 Chimeric Fab

The heavy chain and light chain variable regions from the hybridoma N19-8 were cloned by PCR using the Ig-Prime System (Novagen) as described by the manufacturer. Clones from multiple independent PCR reactions were sequenced to detect mutations introduced during the PCR amplification. An N19-8 VL/human kappa constant region chimeric cDNA was created by using a plasmid containing the N19-8 light chain variable region and the plasmid pHuCK (Hieter et al., 1980 Cell, 22:197–207) as templates in an overlapping PCR reaction.

Similarly, an N19-8 VH/human IgG1 Fd chimeric cDNA was created using a plasmid containing the N19-8 heavy chain variable region and a plasmid containing the human IgG1 gene (obtained from Ilan R. Kirsch, National Cancer Institute, Bethesda, Md.) as templates. This Fd construct contained the first nine amino acids of the IgG1 hinge region, including the cysteine residue which normally forms a disulfide bond with the terminal cysteine residue of the kappa light chain.

The resulting chimeric cDNAs were separately cloned into the APEX-1 vector using appropriate flanking restriction enzyme sites introduced during the PCR amplification procedure and sequenced. A fragment containing the promoter, intron, and cDNA insert from one of these APEX vectors was subsequently subcloned into the polylinker of the other to produce a single vector directing the expression of both the light chain and Fd. The tandem expression cassette from this APEX-1 vector was subsequently subcloned into APEX-3P, which was transfected into 293 EBNA cells for expression of the chimeric Fab.

When tested for the ability to block complement hemolytic activity and C5a generation, the chimeric N19/8 Fab retained the ability to block hemolytic activity, but lost 50% of its C5a generation blocking capacity.

Throughout this application various publications and patent disclosures are referred to. The teachings and disclosures thereof, in their entireties, are hereby incorporated by reference into this application to more fully describe the state of the art to which the present invention pertains.

Although preferred and other embodiments of the invention have been described herein, further embodiments may be perceived by those skilled in the art without departing from the scope of the invention as defined by the following claims.

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TABLE 1

Prevention/Reduction of Proteinuria by Treatment With Anti-C5 Antibodies

	Before Treatment Urine Protein (mg/dL)	After Treatment Urine Protein (mg/dL)
	PBS Control	
mouse A	none	100
mouse B	none	500
mouse C	none	500
mouse D*	trace	trace
mouse E	100	100
	Anti-C5 Treated	
mouse 1	none	none
mouse 2	none	30
mouse 3	30	trace
mouse 4	30	30
mouse 5	30	30
mouse 6	100	30

*Mouse D had more than 500 mg/dL urine glucose after treatment

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 26

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: Amino Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE:
(A) DESCRIPTION: KSSKC peptide

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Val Ile Asp His Gln Gly Thr Lys Ser Ser
5 10

Lys Cys Val Arg Gln Lys Val Glu Gly Ser Ser
15 20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1676 Amino Acids
(B) TYPE: Amino Acid

-continued

(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE:
(A) DESCRIPTION: Pro-C5 Polytpeptide

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Haviland, D.L.
Haviland, J.C.
Fleischer, D.T.
Hunt, A.
Wetsel, R.A.
(B) TITLE: Complete cDNA Sequence of Human Complement Pro-C5
(C) JOURNAL: Journal of Immunology
(D) VOLUME: 146
(F) PAGES: 362-368
(G) DATE: 1991

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Gly Leu Leu Gly Ile Leu Cys Phe Leu
-15                 -10

Ile Phe Leu Gly Lys Thr Trp Gly Gln Glu Gln Thr Tyr Val
-5              -1                   5

Ile Ser Ala Pro Lys Ile Phe Arg Val Gly Ala Ser Glu Asn
10                  15                  20

Ile Val Ile Gln Val Tyr Gly Tyr Thr Glu Ala Phe Asp Ala
25                  30

Thr Ile Ser Ile Lys Ser Tyr Pro Asp Lys Lys Phe Ser Tyr
35                  40                  45

Ser Ser Gly His Val His Leu Ser Ser Glu Asn Lys Phe Gln
50                  55                  60

Asn Ser Ala Ile Leu Thr Ile Gln Pro Lys Gln Leu Pro Gly
65                  70                  75

Gly Gln Asn Pro Val Ser Tyr Val Tyr Leu Glu Val Val Ser
80                  85                  90

Lys His Phe Ser Lys Ser Lys Arg Met Pro Ile Thr Tyr Asp
95                 100

Asn Gly Phe Leu Phe Ile His Thr Asp Lys Pro Val Tyr Thr
105                 110                 115

Pro Asp Gln Ser Val Lys Val Arg Val Tyr Ser Leu Asn Asp
120                 125                 130

Asp Leu Lys Pro Ala Lys Arg Glu Thr Val Leu Thr Phe Ile
135                 140                 145

Asp Pro Glu Gly Ser Glu Val Asp Met Val Glu Glu Ile Asp
150                 155                 160

His Ile Gly Ile Ile Ser Phe Pro Asp Phe Lys Ile Pro Ser
165                 170

Asn Pro Arg Tyr Gly Met Trp Thr Ile Lys Ala Lys Tyr Lys
175                 180                 185

Glu Asp Phe Ser Thr Thr Gly Thr Ala Tyr Phe Glu Val Lys
190                 195                 200

Glu Tyr Val Leu Pro His Phe Ser Val Ser Ile Glu Pro Glu
205                 210                 215
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Tyr Asn Phe Ile Gly Tyr Lys Asn Phe Lys Asn Phe Glu Ile
220                 225                 230

Thr Ile Lys Ala Arg Tyr Phe Tyr Asn Lys Val Val Thr Glu
235                 240

Ala Asp Val Tyr Ile Thr Phe Gly Ile Arg Glu Asp Leu Lys
245                 250                 255

Asp Asp Gln Lys Glu Met Met Gln Thr Ala Met Gln Asn Thr
260                 265                 270

Met Leu Ile Asn Gly Ile Ala Gln Val Thr Phe Asp Ser Glu
275                 280                 285

Thr Ala Val Lys Glu Leu Ser Tyr Tyr Ser Leu Glu Asp Leu
290                 295                 300

Asn Asn Lys Tyr Leu Tyr Ile Ala Val Thr Val Ile Glu Ser
305                 310

Thr Gly Gly Phe Ser Glu Glu Ala Glu Ile Pro Gly Ile Lys
315                 320                 325

Tyr Val Leu Ser Pro Tyr Lys Leu Asn Leu Val Ala Thr Pro
330                 335                 340

Leu Phe Leu Lys Pro Gly Ile Pro Tyr Pro Ile Lys Val Gln
345                 350                 355

Val Lys Asp Ser Leu Asp Gln Leu Val Gly Gly Val Pro Val
360                 365                 370

Ile Leu Asn Ala Gln Thr Ile Asp Val Asn Gln Glu Thr Ser
375                 380

Asp Leu Asp Pro Ser Lys Ser Val Thr Arg Val Asp Asp Gly
385                 390                 395

Val Ala Ser Phe Val Leu Asn Leu Pro Ser Gly Val Thr Val
400                 405                 410

Leu Glu Phe Asn Val Lys Thr Asp Ala Pro Asp Leu Pro Glu
415                 420                 425

Glu Asn Gln Ala Arg Glu Gly Tyr Arg Ala Ile Ala Tyr Ser
430                 435                 440

Ser Leu Ser Gln Ser Tyr Leu Tyr Ile Asp Trp Thr Asp Asn
445                 450

His Lys Ala Leu Leu Val Gly Glu His Leu Asn Ile Ile Val
455                 460                 465

Thr Pro Lys Ser Pro Tyr Ile Asp Lys Ile Thr His Tyr Asn
470                 475                 480

Tyr Leu Ile Leu Ser Lys Gly Lys Ile Ile His Phe Gly Thr
485                 490                 495

Arg Glu Lys Phe Ser Asp Ala Ser Tyr Gln Ser Ile Asn Ile
500                 505                 510

Pro Val Thr Gln Asn Met Val Pro Ser Ser Arg Leu Leu Val
515                 520

Tyr Tyr Ile Val Thr Gly Glu Gln Thr Ala Glu Leu Val Ser
525                 530                 535

Asp Ser Val Trp Leu Asn Ile Glu Glu Lys Cys Gly Asn Gln
540                 545                 550

Leu Gln Val His Leu Ser Pro Asp Ala Asp Ala Tyr Ser Pro
555                 560                 565

Gly Gln Thr Val Ser Leu Asn Met Ala Thr Gly Met Asp Ser
570                 575                 580
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Trp Val Ala Leu Ala Ala Val Asp Ser Ala Val Tyr Gly Val
585                 590

Gln Arg Gly Ala Lys Lys Pro Leu Glu Arg Val Phe Gln Phe
595                 600                 605

Leu Glu Lys Ser Asp Leu Gly Cys Gly Ala Gly Gly Gly Leu
610                 615                 620

Asn Asn Ala Asn Val Phe His Leu Ala Gly Leu Thr Phe Leu
625                 630                 635

Thr Asn Ala Asn Ala Asp Asp Ser Gln Glu Asn Asp Glu Pro
640                 645                 650

Cys Lys Glu Ile Leu Arg Pro Arg Arg Thr Leu Gln Lys Lys
655                 660

Ile Glu Glu Ile Ala Ala Lys Tyr Lys His Ser Val Val Lys
665                 670                 675

Lys Cys Cys Tyr Asp Gly Ala Cys Val Asn Asn Asp Glu Thr
680                 685                 690

Cys Glu Gln Arg Ala Ala Arg Ile Ser Leu Gly Pro Arg Cys
695                 700                 705

Ile Lys Ala Phe Thr Glu Cys Cys Val Val Ala Ser Gln Leu
710                 715                 720

Arg Ala Asn Ile Ser His Lys Asp Met Gln Leu Gly Arg Leu
725                 730

His Met Lys Thr Leu Leu Pro Val Ser Lys Pro Glu Ile Arg
735                 740                 745

Ser Tyr Phe Pro Glu Ser Trp Leu Trp Glu Val His Leu Val
750                 755                 760

Pro Arg Arg Lys Gln Leu Gln Phe Ala Leu Pro Asp Ser Leu
765                 770                 775

Thr Thr Trp Glu Ile Gln Gly Ile Gly Ile Ser Asn Thr Gly
780                 785                 790

Ile Cys Val Ala Asp Thr Val Lys Ala Lys Val Phe Lys Asp
795                 800

Val Phe Leu Glu Met Asn Ile Pro Tyr Ser Val Val Arg Gly
805                 810                 815

Glu Gln Ile Gln Leu Lys Gly Thr Val Tyr Asn Tyr Arg Thr
820                 825                 830

Ser Gly Met Gln Phe Cys Val Lys Met Ser Ala Val Glu Gly
835                 840                 845

Ile Cys Thr Ser Glu Ser Pro Val Ile Asp His Gln Gly Thr
850                 855                 860

Lys Ser Ser Lys Cys Val Arg Gln Lys Val Glu Gly Ser Ser
865                 870

Ser His Leu Val Thr Phe Thr Val Leu Pro Leu Glu Ile Gly
875                 880                 885

Leu His Asn Ile Asn Phe Ser Leu Glu Thr Trp Phe Gly Lys
890                 895                 900

Glu Ile Leu Val Lys Thr Leu Arg Val Val Pro Glu Gly Val
905                 910                 915

Lys Arg Glu Ser Tyr Ser Gly Val Thr Leu Asp Pro Arg Gly
920                 925                 930

Ile Tyr Gly Thr Ile Ser Arg Arg Lys Glu Phe Pro Tyr Arg
935                 940
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Ile Pro Leu Asp Leu Val Pro Lys Thr Glu Ile Lys Arg Ile
945                 950                 955

Leu Ser Val Lys Gly Leu Leu Val Gly Glu Ile Leu Ser Ala
960                 965                 970

Val Leu Ser Gln Glu Gly Ile Asn Ile Leu Thr His Leu Pro
975                 980                 985

Lys Gly Ser Ala Glu Ala Glu Leu Met Ser Val Val Pro Val
990                 995                 1000

Phe Tyr Val Phe His Tyr Leu Glu Thr Gly Asn His Trp Asn
1005                1010

Ile Phe His Ser Asp Pro Leu Ile Glu Lys Gln Lys Leu Lys
1015                1020                1025

Lys Lys Leu Lys Glu Gly Met Leu Ser Ile Met Ser Tyr Arg
1030                1035                1040

Asn Ala Asp Tyr Ser Tyr Ser Val Trp Lys Gly Gly Ser Ala
1045                1050                1055

Ser Thr Trp Leu Thr Ala Phe Ala Leu Arg Val Leu Gly Gln
1060                1065                1070

Val Asn Lys Tyr Val Glu Gln Asn Gln Asn Ser Ile Cys Asn
1075                1080

Ser Leu Leu Trp Leu Val Glu Asn Tyr Gln Leu Asp Asn Gly
1085                1090                1095

Ser Phe Lys Glu Asn Ser Gln Tyr Gln Pro Ile Lys Leu Gln
1100                1105                1110

Gly Thr Leu Pro Val Glu Ala Arg Glu Asn Ser Leu Tyr Leu
1115                1120                1125

Thr Ala Phe Thr Val Ile Gly Ile Arg Lys Ala Phe Asp Ile
1130                1135                1140

Cys Pro Leu Val Lys Ile Asp Thr Ala Leu Ile Lys Ala Asp
1145                1150

Asn Phe Leu Leu Glu Asn Thr Leu Pro Ala Gln Ser Thr Phe
1155                1160                1165

Thr Leu Ala Ile Ser Ala Tyr Ala Leu Ser Leu Gly Asp Lys
1170                1175                1180

Thr His Pro Gln Phe Arg Ser Ile Val Ser Ala Leu Lys Arg
1185                1190                1195

Glu Ala Leu Val Lys Gly Asn Pro Pro Ile Tyr Arg Phe Trp
1200                1205                1210

Lys Asp Asn Leu Gln His Lys Asp Ser Ser Val Pro Asn Thr
1215                1220

Gly Thr Ala Arg Met Val Glu Thr Thr Ala Tyr Ala Leu Leu
1225                1230                1235

Thr Ser Leu Asn Leu Lys Asp Ile Asn Tyr Val Asn Pro Val
1240                1245                1250

Ile Lys Trp Leu Ser Glu Glu Gln Arg Tyr Gly Gly Gly Phe
1255                1260                1265

Tyr Ser Thr Gln Asp Thr Ile Asn Ala Ile Glu Gly Leu Thr
1270                1275                1280

Glu Tyr Ser Leu Leu Val Lys Gln Leu Arg Leu Ser Met Asp
1285                1290

Ile Asp Val Ser Tyr Lys His Lys Gly Ala Leu His Asn Tyr
1295                1300                1305
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Lys Met Thr Asp Lys Asn Phe Leu Gly Arg Pro Val Glu Val
1310                1315                1320

Leu Leu Asn Asp Asp Leu Ile Val Ser Thr Gly Phe Gly Ser
1325                1330                1335

Gly Leu Ala Thr Val His Val Thr Thr Val Val His Lys Thr
1340                1345                1350

Ser Thr Ser Glu Glu Val Cys Ser Phe Tyr Leu Lys Ile Asp
1355                1360

Thr Gln Asp Ile Glu Ala Ser His Tyr Arg Gly Tyr Gly Asn
1365                1370                1375

Ser Asp Tyr Lys Arg Ile Val Ala Cys Ala Ser Tyr Lys Pro
1380                1385                1390

Ser Arg Glu Glu Ser Ser Ser Gly Ser Ser His Ala Val Met
1395                1400                1405

Asp Ile Ser Leu Pro Thr Gly Ile Ser Ala Asn Glu Glu Asp
1410                1415                1420

Leu Lys Ala Leu Val Glu Gly Val Asp Gln Leu Phe Thr Asp
1425                1430

Tyr Gln Ile Lys Asp Gly His Val Ile Leu Gln Leu Asn Ser
1435                1440                1445

Ile Pro Ser Ser Asp Phe Leu Cys Val Arg Phe Arg Ile Phe
1450                1455                1460

Glu Leu Phe Glu Val Gly Phe Leu Ser Pro Ala Thr Phe Thr
1465                1470                1475

Val Tyr Glu Tyr His Arg Pro Asp Lys Gln Cys Thr Met Phe
1480                1485                1490

Tyr Ser Thr Ser Asn Ile Lys Ile Gln Lys Val Cys Glu Gly
1495                1500

Ala Ala Cys Lys Cys Val Glu Ala Asp Cys Gly Gln Met Gln
1505                1510                1515

Glu Glu Leu Asp Leu Thr Ile Ser Ala Glu Thr Arg Lys Gln
1520                1525                1530

Thr Ala Cys Lys Pro Glu Ile Ala Tyr Ala Tyr Lys Val Ser
1535                1540                1545

Ile Thr Ser Ile Thr Val Glu Asn Val Phe Val Lys Tyr Lys
1550                1555                1560

Ala Thr Leu Leu Asp Ile Tyr Lys Thr Gly Glu Ala Val Ala
1565                1570

Glu Lys Asp Ser Glu Ile Thr Phe Ile Lys Lys Val Thr Cys
1575                1580                1585

Thr Asn Ala Glu Leu Val Lys Gly Arg Gln Tyr Leu Ile Met
1590                1595                1600

Gly Lys Glu Ala Leu Gln Ile Lys Tyr Asn Phe Ser Phe Arg
1605                1610                1615

Tyr Ile Tyr Pro Leu Asp Ser Leu Thr Trp Ile Glu Tyr Trp
1620                1625                1630

Pro Arg Asp Thr Thr Cys Ser Ser Cys Gln Ala Phe Leu Ala
1635                1640

Asn Leu Asp Glu Phe Ala Glu Asp Ile Phe Leu Asn Gly Cys
1645                1650                1655
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4059 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: Circular

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Apex-1 Eukaryotic Expression Vector

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACGCGTTGAC ATTGATTATT GACTAGTTAT TAATAGTAAT CAATTACGGG 50

GTCATTAGTT CATAGCCCAT ATATGGAGTT CCGCGTTACA TAACTTACGG 100

TAAATGGCCC CGCCTGGCTG ACCGCCCAAC GACCCCCGCC CATTGACGTC 150

AATAATGACG TATGTTCCCA TAGTAACGCC AATAGGGACT TTCCATTGAC 200

GTCAATGGGT GGACTATTTA CGGTAAACTG CCCACTTGGC AGTACATCAA 250

GTGTATCATA TGCCAAGTAC GCCCCCTATT GACGTCAATG ACGGTAAATG 300

GCCCGCCTGG CATTATGCCC AGTACATGAC CTTATGGGAC TTTCCTACTT 350

GGCAGTACAT CTACGTATTA GTCATCGCTA TTACCATGGT GATGCGGTTT 400

TGGCAGTACA TCAATGGGCG TGGATAGCGG TTTGACTCAC GGGGATTTCC 450

AAGTCTCCAC CCCATTGACG TCAATGGGAG TTTGTTTTGG CACCAAAATC 500

AACGGGACTT TCCAAAATGT CGTAACAACT CCGCCCCATT GACGCAAATG 550

GGCGGTAGGC GTGTACGGTG GGAGGTCTAT ATAAGCAGAG CTCGTTTAGT 600

GAACCGTCAG AATTCTGTTG GGCTCGCGGT TGATTACAAA CTCTTCGCGG 650

TCTTTCCAGT ACTCTTGGAT CGGAAACCCG TCGGCCTCCG AACGGTACTC 700

CGCCACCGAG GGACCTGAGC GAGTCCGCAT CGACCGGATC GGAAAACCTC 750

TCGACTGTTG GGGTGAGTAC TCCCTCTCAA AAGCGGGCAT GACTTCTGCG 800

CTAAGATTGT CAGTTTCCAA AAACGAGGAG GATTTGATAT TCACCTGGCC 850

CGCGGTGATG CCTTTGAGGG TGGCCGCGTC CATCTGGTCA GAAAAGACAA 900

TCTTTTTGTT GTCAAGCTTG AGGTGTGGCA GGCTTGAGAT CTGGCCATAC 950

ACTTGAGTGA CAATGACATC CACTTTGCCT TTCTCTCCAC AGGTGTCCAC 1000

TCCCAGGTCC AACTGCAGGT CGACCGGCTT GGTACCGAGC TCGGATCCAC 1050

TAGTAACGGC CGCCAGTGTG CTGGAATTCT GCAGATATCC ATCACACTGG 1100

CGGCCGCTCG AGCATGCATC TAGAACTTGT TTATTGCAGC TTATAATGGT 1150

TACAAATAAA GCAATAGCAT CACAAATTTC ACAAATAAAG CATTTTTTTC 1200

ACTGCATTCT AGTTGTGGTT TGTCCAAACT CATCAATGTA TCTTATCATG 1250

TCTGGATCGA TCCCGCCATG GTATCAACGC CATATTTCTA TTTACAGTAG 1300

GGACCTCTTC GTTGTGTAGG TACCGCTGTA TTCCTAGGGA AATAGTAGAG 1350

GCACCTTGAA CTGTCTGCAT CAGCCATATA GCCCCCGCTG TTCGACTTAC 1400

AAACACAGGC ACAGTACTGA CAAACCCATA CACCTCCTCT GAAATACCCA 1450

TAGTTGCTAG GGCTGTCTCC GAACTCATTA CACCCTCCAA AGTCAGAGCT 1500

GTAATTTCGC CATCAAGGGC AGCGAGGGCT TCTCCAGATA AAATAGCTTC 1550

TGCCGAGAGT CCCGTAAGGG TAGACACTTC AGCTAATCCC TCGATGAGGT 1600

CTACTAGAAT AGTCAGTGCG GCTCCCATTT TGAAAATTCA CTTACTTGAT 1650

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CAGCTTCAGA AGATGGCGGA GGGCCTCCAA CACAGTAATT TTCCTCCCGA	1700
CTCTTAAAAT AGAAAATGTC AAGTCAGTTA AGCAGGAAGT GGACTAACTG	1750
ACGCAGCTGG CCGTGCGACA TCCTCTTTTA ATTAGTTGCT AGGCAACGCC	1800
CTCCAGAGGG CGTGTGGTTT TGCAAGAGGA AGCAAAAGCC TCTCCACCCA	1850
GGCCTAGAAT GTTTCCACCC AATCATTACT ATGACAACAG CTGTTTTTTT	1900
TAGTATTAAG CAGAGGCCGG GGACCCCTGG GCCCGCTTAC TCTGGAGAAA	1950
AAGAAGAGAG GCATTGTAGA GGCTTCCAGA GGCAACTTGT CAAAACAGGA	2000
CTGCTTCTAT TTCTGTCACA CTGTCTGGCC CTGTCACAAG GTCCAGCACC	2050
TCCATACCCC CTTTAATAAG CAGTTTGGGA ACGGGTGCGG GTCTTACTCC	2100
GCCCATCCCG CCCCTAACTC CGCCCAGTTC CGCCCATTCT CCGCCCCATG	2150
GCTGACTAAT TTTTTTTATT TATGCAGAGG CCGAGGCCGC CTCGGCCTCT	2200
GAGCTATTCC AGAAGTAGTG AGGAGGCTTT TTTGGAGGCC TAGGCTTTTG	2250
CAAAAAGGAG CTCCCAGCAA AAGGCCAGGA ACCGTAAAAA GGCCGCGTTG	2300
CTGGCGTTTT TCCATAGGCT CCGCCCCCCT GACGAGCATC ACAAAAATCG	2350
ACGCTCAAGT CAGAGGTGGC GAAACCCGAC AGGACTATAA AGATACCAGG	2400
CGTTTCCCCC TGGAAGCTCC CTCGTGCGCT CTCCTGTTCC GACCCTGCCG	2450
CTTACCGGAT ACCTGTCCGC CTTTCTCCCT TCGGGAAGCG TGGCGCTTTC	2500
TCAATGCTCA CGCTGTAGGT ATCTCAGTTC GGTGTAGGTC GTTCGCTCCA	2550
AGCTGGGCTG TGTGCACGAA CCCCCCGTTC AGCCCGACCG CTGCGCCTTA	2600
TCCGGTAACT ATCGTCTTGA GTCCAACCCG GTAAGACACG ACTTATCGCC	2650
ACTGGCAGCA GCCACTGGTA ACAGGATTAG CAGAGCGAGG TATGTAGGCG	2700
GTGCTACAGA GTTCTTGAAG TGGTGGCCTA ACTACGGCTA CACTAGAAGG	2750
ACAGTATTTG GTATCTGCGC TCTGCTGAAG CCAGTTACCT TCGGAAAAAG	2800
AGTTGGTAGC TCTTGATCCG GCAAACAAAC CACCGCTGGT AGCGGTGGTT	2850
TTTTTGTTTG CAAGCAGCAG ATTACGCGCA GAAAAAAAGG ATCTCAAGAA	2900
GATCCTTTGA TCTTTTCTAC GGGGTCTGAC GCTCAGTGGA ACGAAAACTC	2950
ACGTTAAGGG ATTTTGGTCA TGAGATTATC AAAAAGGATC TTCACCTAGA	3000
TCCTTTTAAA TTAAAAATGA AGTTTTAAAT CAATCTAAAG TATATATGAG	3050
TAAACTTGGT CTGACAGTTA CCAATGCTTA ATCAGTGAGG CACCTATCTC	3100
AGCGATCTGT CTATTTCGTT CATCCATAGT TGCCTGACTC CCCGTCGTGT	3150
AGATAACTAC GATACGGGAG GGCTTACCAT CTGGCCCCAG TGCTGCAATG	3200
ATACCGCGAG ACCCACGCTC ACCGGCTCCA GATTTATCAG CAATAAACCA	3250
GCCAGCCGGA AGGGCCGAGC GCAGAAGTGG TCCTGCAACT TTATCCGCCT	3300
CCATCCAGTC TATTAATTGT TGCCGGGAAG CTAGAGTAAG TAGTTCGCCA	3350
GTTAATAGTT TGCGCAACGT TGTTGCCATT GCTACAGGCA TCGTGGTGTC	3400
ACGCTCGTCG TTTGGTATGG CTTCATTCAG CTCCGGTTCC CAACGATCAA	3450
GGCGAGTTAC ATGATCCCCC ATGTTGTGCA AAAAAGCGGT TAGCTCCTTC	3500
GGTCCTCCGA TCGTTGTCAG AAGTAAGTTG GCCGCAGTGT TATCACTCAT	3550
GGTTATGGCA GCACTGCATA ATTCTCTTAC TGTCATGCCA TCCGTAAGAT	3600
GCTTTTCTGT GACTGGTGAG TACTCAACCA AGTCATTCTG AGAATAGTGT	3650

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ATGCGGCGAC CGAGTTGCTC TTGCCCGGCG TCAATACGGG ATAATACCGC 3700

GCCACATAGC AGAACTTTAA AAGTGCTCAT CATTGGAAAA CGTTCTTCGG 3750

GGCGAAAACT CTCAAGGATC TTACCGCTGT TGAGATCCAG TTCGATGTAA 3800

CCCACTCGTG CACCCAACTG ATCTTCAGCA TCTTTTACTT TCACCAGCGT 3850

TTCTGGGTGA GCAAAAACAG GAAGGCAAAA TGCCGCAAAA AAGGGAATAA 3900

GGGCGACACG GAAATGTTGA ATACTCATAC TCTTCCTTTT TCAATATTAT 3950

TGAAGCATTT ATCAGGGTTA TTGTCTCATG AGCGGATACA TATTTGAATG 4000

TATTTAGAAA AATAAACAAA TAGGGGTTCC GCGCACATTT CCCCGAAAAG 4050

TGCCACCTG 4059

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8540 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: Circular

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Apex-3P Eukaryotic Expression Vector.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTGACCAATA CAAAACAAAA GCGCCCCTCG TACCAGCGAA GAAGGGGCAG 50

AGATGCCGTA GTCAGGTTTA GTTCGTCCGG CGGCGGGGGA TCTGTATGGT 100

GCACTCTCAG TACAATCTGC TCTGATGCCG CATAGTTAAG CCAGTATCTG 150

CTCCCTGCTT GTGTGTTGGA GGTCGCTGAG TAGTGCGCGA GCAAAATTTA 200

AGCTACAACA AGGCAAGGCT TGACCGACAA TTGCATGAAG AATCTGCTTA 250

GGGTTAGGCG TTTTGCGCTG CTTCGCGATG TACGGGCCAG ATATACGCGT 300

TGACATTGAT TATTGACTAG TTATTAATAG TAATCAATTA CGGGGTCATT 350

AGTTCATAGC CCATATATGG AGTTCCGCGT TACATAACTT ACGGTAAATG 400

GCCCGCCTGG CTGACCGCCC AACGACCCCC GCCCATTGAC GTCAATAATG 450

ACGTATGTTC CCATAGTAAC GCCAATAGGG ACTTTCCATT GACGTCAATG 500

GGTGGACTAT TTACGGTAAA CTGCCCACTT GGCAGTACAT CAAGTGTATC 550

ATATGCCAAG TACGCCCCCT ATTGACGTCA ATGACGGTAA ATGGCCCGCC 600

TGGCATTATG CCCAGTACAT GACCTTATGG GACTTTCCTA CTTGGCAGTA 650

CATCTACGTA TTAGTCATCG CTATTACCAT GGTGATGCGG TTTTGGCAGT 700

ACATCAATGG GCGTGGATAG CGGTTTGACT CACGGGGATT TCCAAGTCTC 750

CACCCCATTG ACGTCAATGG GAGTTTGTTT TGGCACCAAA ATCAACGGGA 800

CTTTCCAAAA TGTCGTAACA ACTCCGCCCC ATTGACGCAA ATGGGCGGTA 850

GGCGTGTACG GTGGGAGGTC TATATAAGCA GAGCTCGTTT AGTGAACCGT 900

CAGAATTCTG TTGGGCTCGC GGTTGATTAC AAACTCTTCG CGGTCTTTCC 950

AGTACTCTTG GATCGGAAAC CCGTCGGCCT CCGAACGGTA CTCCGCCACC 1000

GAGGGACCTG AGCGAGTCCG CATCGACCGG ATCGGAAAAC CTCTCGACTG 1050

TTGGGGTGAG TACTCCCTCT CAAAAGCGGG CATGACTTCT GCGCTAAGAT 1100

TGTCAGTTTC CAAAAACGAG GAGGATTTGA TATTCACCTG GCCCGCGGTG 1150

ATGCCTTTGA GGGTGGCCGC GTCCATCTGG TCAGAAAAGA CAATCTTTTT 1200

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GTTGTCAAGC TTGAGGTGTG GCAGGCTTGA GATCTGGCCA TACACTTGAG	1250
TGACAATGAC ATCCACTTTG CCTTTCTCTC CACAGGTGTC CACTCCCAGG	1300
TCCAACTGCA GGTCGACCGG CTTGGTACCG AGCTCGGATC CTCTAGAGTC	1350
GACCTGCAGG CATGCAAGCT TGGCACTGGC CGTCGTTTTA CAACGTCGTG	1400
ACTGGGAAAA CCCTGGCGTT ACCCAACTTA ATCGCCTTGC AGCACATCCC	1450
CCTTTCGCCA GCTGGCGTAA TAGCGAAGAG GCCCGCACCG ATCCAGACAT	1500
GATAAGATAC ATTGATGAGT TTGGACAAAC CACAACTAGA ATGCAGTGAA	1550
AAAAATGCTT TATTTGTGAA ATTTGTGATG CTATTGCTTT ATTTGTAACC	1600
ATTATAAGCT GCAATAAACA AGTTAACAAC AACAATTGCA TTCATTTTAT	1650
GTTTCAGGTT CAGGGGGAGG TGTGGGAGGT TTTTTAAAGC AAGTAAAACC	1700
TCTACAAATG TGGTATGGCT GATTATGATC CCCAGGAAGC TCCTCTGTGT	1750
CCTCATAAAC CCTAACCTCC TCTACTTGAG AGGACATTCC AATCATAGGC	1800
TGCCCATCCA CCCTCTGTGT CCTCCTGTTA ATTAGGTCAC TTAACAAAAA	1850
GGAAATTGGG TAGGGGTTTT TCACAGACCG CTTTCTAAGG GTAATTTTAA	1900
AATATCTGGG AAGTCCCTTC CACTGCTGTG TTCCAGAAGT GTTGGTAAAC	1950
AGCCCACAAA TGTCAACAGC AGAAACATAC AAGCTGTCAG CTTTGCACAA	2000
GGGCCCAACA CCCTGCTCAT CAAGAAGCAC TGTGGTTGCT GTGTTAGTAA	2050
TGTGCAAAAC AGGAGGCACA TTTTCCCCAC CTGTGTAGGT TCCAAAATAT	2100
CTAGTGTTTT CATTTTTACT TGGATCAGGA ACCCAGCACT CCACTGGATA	2150
AGCATTATCC TTATCCAAAA CAGCCTTGTG GTCAGTGTTC ATCTGCTGAC	2200
TGTCAACTGT AGCATTTTTT GGGGTTACAG TTTGAGCAGG ATATTTGGTC	2250
CTGTAGTTTG CTAACACACC CTGCAGCTCC AAAGGTTCCC CACCAACAGC	2300
AAAAAAATGA AAATTTGACC CTTGAATGGG TTTTCCAGCA CCATTTTCAT	2350
GAGTTTTTTG TGTCCCTGAA TGCAAGTTTA ACATAGCAGT TACCCCAATA	2400
ACCTCAGTTT TAACAGTAAC AGCTTCCCAC ATCAAAATAT TTCCACAGGT	2450
TAAGTCCTCA TTTGTAGAAT TCGCCAGCAC AGTGGTCGAC CCTGTGGATG	2500
TGTGTCACTT AGGGTGTGGA AAGTCCCCAG GCTCCCCAGC AGGCAGAAGT	2550
ATGCAAAGCA TGCATCTCAA TTAGTCAGCA ACCAGGTGTG GAAAGTCCCC	2600
AGGCTCCCCA GCAGGCAGAA GTATGCAAAG CATGCATCTC AATTAGTCAG	2650
CAACCATAGT CCCGCCCCTA ACTCCGCCCA TCCCGCCCCT AACTCCGCCC	2700
AGTTCCGCCC ATTCTCCGCC CCATGGCTGA CTAATTTTTT TTATTTATGC	2750
AGAGGCCGAG GCCGCCTCGG CCTCTGAGCT ATTCCAGAAG TAGTGAGGAG	2800
GCTTTTTTGG AGGCCTAGGC TTTTGCAAAA GCTTACCATG ACCGAGTACA	2850
AGCCCACGGT GCGCCTCGCC ACCCGCGACG ACGTCCCCCG GGCCGTACGC	2900
ACCCTCGCCG CCGCGTTCGC CGACTACCCC GCCACGCGCC ACACCGTCGA	2950
CCCGGACCGC CACATCGAGC GGGTCACCGA GCTGCAAGAA CTCTTCCTCA	3000
CGCGCGTCGG GCTCGACATC GGCAAGGTGT GGGTCGCGGA CGACGGCGCC	3050
GCGGTGGCGG TCTGGACCAC GCCGGAGAGC GTCGAAGCGG GGGCGGTGTT	3100
CGCCGAGATC GGCCCGCGCA TGGCCGAGTT GAGCGGTTCC CGGCTGGCCG	3150
CGCAGCAACA GATGGAAGGC CTCCTGGCGC CGCACCGGCC CAAGGAGCCC	3200

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GCGTGGTTCC TGGCCACCGT CGGCGTCTCG CCCGACCACC AGGGCAAGGG	3250
TCTGGGCAGC GCCGTCGTGC TCCCCGGAGT GGAGGCGGCC GAGCGCGCCG	3300
GGGTGCCCGC CTTCCTGGAG ACCTCCGCGC CCCGCAACCT CCCCTTCTAC	3350
GAGCGGCTCG GCTTCACCGT CACCGCCGAC GTCGAGTGCC CGAAGGACCG	3400
CGCGACCTGG TGCATGACCC GCAAGCCCGG TGCCTGACGC CCGCCCCACG	3450
ACCCGCAGCG CCCGACCGAA AGGAGCGCAC GACCCCATGC ATCGATAAAA	3500
TAAAAGATTT TATTTAGTCT CCAGAAAAAG GGGGGAATGA AAGACCCCAC	3550
CTGTAGGTTT GGCAAGCTAG AACTTGTTTA TTGCAGCTTA TAATGGTTAC	3600
AAATAAAGCA ATAGCATCAC AAATTTCACA AATAAAGCAT TTTTTTCACT	3650
GCATTCTAGT TGTGGTTTGT CCAAACTCAT CAATGTATCT TATCATGTCT	3700
GGATCGATCC CGCCATGGTA TCAACGCCAT ATTTCTATTT ACAGTAGGGA	3750
CCTCTTCGTT GTGTAGGTAC CCCGGGTTCG AAATCGAATT CGCCAATGAC	3800
AAGACGCTGG GCGGGGTTTG TGTCATCATA GAACTAAAGA CATGCAAATA	3850
TATTTCTTCC GGGGACACCG CCAGCAAACG CGAGCAACGG GCCACGGGGA	3900
TGAAGCAGCC CGGCGGCACC TCGCTAACGG ATTCACCACT CCAAGAATTG	3950
GAGCCAATCA ATTCTTGCGG AGAACTGTGA ATGCGCAAAC CAACCCTTGG	4000
CAGAACATAT CCATCGCGTC CGCCATCTCC AGCAGCCGCA CGCGGCGCAT	4050
CTCGGGGCCG ACGCGCTGGG CTACGTCTTG CTGGCGTTCG CGACGCGAGG	4100
CTGGATGGCC TTCCCCATTA TGATTCTTCT CGCTTCCGGC GGCATCGGGA	4150
TGCCCGCGTT GCAGGCCATG CTGTCCAGGC AGGTAGATGA CGACCATCAG	4200
GGACAGCTTC AAGGATCGCT CGCGGCTCTT ACCAGCGCCA GCAAAAGGCC	4250
AGGAACCGTA AAAAGGCCGC GTTGCTGGCG TTTTTCCATA GGCTCCGCCC	4300
CCCTGACGAG CATCACAAAA ATCGACGCTC AAGTCAGAGG TGGCGAAACC	4350
CGACAGGACT ATAAAGATAC CAGGCGTTTC CCCCTGGAAG CTCCCTCGTG	4400
CGCTCTCCTG TTCCGACCCT GCCGCTTACC GGATACCTGT CCGCCTTTCT	4450
CCCTTCGGGA AGCGTGGCGC TTTCTCATAG CTCACGCTGT AGGTATCTCA	4500
GTTCGGTGTA GGTCGTTCGC TCCAAGCTGG GCTGTGTGCA CGAACCCCCC	4550
GTTCAGCCCG ACCGCTGCGC CTTATCCGGT AACTATCGTC TTGAGTCCAA	4600
CCCGGTAAGA CACGACTTAT CGCCACTGGC AGCAGCCACT GGTAACAGGA	4650
TTAGCAGAGC GAGGTATGTA GGCGGTGCTA CAGAGTTCTT GAAGTGGTGG	4700
CCTAACTACG GCTACACTAG AAGGACAGTA TTTGGTATCT GCGCTCTGCT	4750
GAAGCCAGTT ACCTTCGGAA AAAGAGTTGG TAGCTCTTGA TCCGGCAAAC	4800
AAACCACCGC TGGTAGCGGT GGTTTTTTTG TTTGCAAGCA GCAGATTACG	4850
CGCAGAAAAA AAGGATCTCA AGAAGATCCT TTGATCTTTT CTACGGGGTC	4900
TGACGCTCAG TGGAACGAAA ACTCACGTTA AGGGATTTTG GTCATGAGAT	4950
TATCAAAAAG GATCTTCACC TAGATCCTTT TAAATTAAAA ATGAAGTTTT	5000
AAATCAATCT AAAGTATATA TGAGTAAACT TGGTCTGACA GTTACCAATG	5050
CTTAATCAGT GAGGCACCTA TCTCAGCGAT CTGTCTATTT CGTTCATCCA	5100
TAGTTGCCTG ACTCCCCGTC GTGTAGATAA CTACGATACG GGAGGGCTTA	5150
CCATCTGGCC CCAGTGCTGC AATGATACCG CGAGACCCAC GCTCACCGGC	5200

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TCCAGATTTA TCAGCAATAA ACCAGCCAGC CGGAAGGGCC GAGCGCAGAA	5250
GTGGTCCTGC AACTTTATCC GCCTCCATCC AGTCTATTAA TTGTTGCCGG	5300
GAAGCTAGAG TAAGTAGTTC GCCAGTTAAT AGTTTGCGCA ACGTTGTTGC	5350
CATTGCTGCA GGCATCGTGG TGTCACGCTC GTCGTTTGGT ATGGCTTCAT	5400
TCAGCTCCGG TTCCCAACGA TCAAGGCGAG TTACATGATC CCCCATGTTG	5450
TGCAAAAAAG CGGTTAGCTC CTTCGGTCCT CCGATCGTTG TCAGAAGTAA	5500
GTTGGCCGCA GTGTTATCAC TCATGGTTAT GGCAGCACTG CATAATTCTC	5550
TTACTGTCAT GCCATCCGTA AGATGCTTTT CTGTGACTGG TGAGTACTCA	5600
ACCAAGTCAT TCTGAGAATA GTGTATGCGG CGACCGAGTT GCTCTTGCCC	5650
GGCGTCAACA CGGGATAATA CCGCGCCACA TAGCAGAACT TTAAAAGTGC	5700
TCATCATTGG AAAACGTTCT TCGGGGCGAA AACTCTCAAG GATCTTACCG	5750
CTGTTGAGAT CCAGTTCGAT GTAACCCACT CGTGCACCCA ACTGATCTTC	5800
AGCATCTTTT ACTTTCACCA GCGTTTCTGG GTGAGCAAAA ACAGGAAGGC	5850
AAAATGCCGC AAAAAAGGGA ATAAGGGCGA CACGGAAATG TTGAATACTC	5900
ATACTCTTCC TTTTTCAATA TTATTGAAGC ATTTATCAGG GTTATTGTCT	5950
CATGAGCGGA TACATATTTG AATGTATTTA GAAAAATAAA CAAATAGGGG	6000
TTCCGCGCAC ATTTCCCCGA AAAGTGCCAC CTGACGTCTA AGAAACCATT	6050
ATTATCATGA CATTAACCTA TAAAAATAGG CGTATCACGA GGCCCTTTCG	6100
TCTTCAAGAA TTCTCATGTT TGACAGCTTA TCGTAGACAT CATGCGTGCT	6150
GTTGGTGTAT TTCTGGCCAT CTGTCTTGTC ACCATTTTCG TCCTCCCAAC	6200
ATGGGGCAAT TGGGCATACC CATGTTGTCA CGTCACTCAG CTCCGCGCTC	6250
AACACCTTCT CGCGTTGGAA AACATTAGCG ACATTTACCT GGTGAGCAAT	6300
CAGACATGCG ACGGCTTTAG CCTGGCCTCC TTAAATTCAC CTAAGAATGG	6350
GAGCAACCAG CAGGAAAAGG ACAAGCAGCG AAAATTCACG CCCCCTTGGG	6400
AGGTGGCGGC ATATGCAAAG GATAGCACTC CCACTCTACT ACTGGGTATC	6450
ATATGCTGAC TGTATATGCA TGAGGATAGC ATATGCTACC CGGATACAGA	6500
TTAGGATAGC ATATACTACC CAGATATAGA TTAGGATAGC ATATGCTACC	6550
CAGATATAGA TTAGGATAGC CTATGCTACC CAGATATAAA TTAGGATAGC	6600
ATATACTACC CAGATATAGA TTAGGATAGC ATATGCTACC CAGATATAGA	6650
TTAGGATAGC CTATGCTACC CAGATATAGA TTAGGATAGC ATATGCTACC	6700
CAGATATAGA TTAGGATAGC ATATGCTATC CAGATATTTG GGTAGTATAT	6750
GCTACCCAGA TATAAATTAG GATAGCATAT ACTACCCTAA TCTCTATTAG	6800
GATAGCATAT GCTACCCGGA TACAGATTAG GATAGCATAT ACTACCCAGA	6850
TATAGATTAG GATAGCATAT GCTACCCAGA TATAGATTAG GATAGCCTAT	6900
GCTACCCAGA TATAAATTAG GATAGCATAT ACTACCCAGA TATAGATTAG	6950
GATAGCATAT GCTACCCAGA TATAGATTAG GATAGCCTAT GCTACCCAGA	7000
TATAGATTAG GATAGCATAT GCTATCCAGA TATTTGGGTA GTATATGCTA	7050
CCCATGGCAA CATTAGCCCA CCGTGCTCTC AGCGACCTCG TGAATATGAG	7100
GACCAACAAC CCTGTGCTTG GCGCTCAGGC GCAAGTGTGT GTAATTTGTC	7150
CTCCAGATCG CAGCAATCGC GCCCCTATCT TGGCCCGCCC ACCTACTTAT	7200

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GCAGGTATTC CCCGGGGTGC CATTAGTGGT TTTGTGGGCA AGTGGTTTGA 7250

CCGCAGTGGT TAGCGGGGTT ACAATCAGCC AAGTTATTAC ACCCTTATTT 7300

TACAGTCCAA AACCGCAGGG CGGCGTGTGG GGGCTGACGC GTGCCCCCAC 7350

TCCACAATTT CAAAAAAAAG AGTGGCCACT TGTCTTTGTT TATGGGCCCC 7400

ATTGGCGTGG AGCCCCGTTT AATTTTCGGG GGTGTTAGAG ACAACCAGTG 7450

GAGTCCGCTG CTGTCGGCGT CCACTCTCTT TCCCCTTGTT ACAAATAGAG 7500

TGTAACAACA TGGTTCACCT GTCTTGGTCC CTGCCTGGGA CACATCTTAA 7550

TAACCCCAGT ATCATATTGC ACTAGGATTA TGTGTTGCCC ATAGCCATAA 7600

ATTCGTGTGA GATGGACATC CAGTCTTTAC GGCTTGTCCC CACCCCATGG 7650

ATTTCTATTG TTAAAGATAT TCAGAATGTT TCATTCCTAC ACTAGTATTT 7700

ATTGCCCAAG GGGTTTGTGA GGGTTATATT GGTGTCATAG CACAATGCCA 7750

CCACTGAACC CCCCGTCCAA ATTTTATTCT GGGGGCGTCA CCTGAAACCT 7800

TGTTTTCGAG CACCTCACAT ACACCTTACT GTTCACAACT CAGCAGTTAT 7850

TCTATTAGCT AAACGAAGGA GAATGAAGAA GCAGGCGAAG ATTCAGGAGA 7900

GTTCACTGCC CGCTCCTTGA TCTTCAGCCA CTGCCCTTGT GACTAAAATG 7950

GTTCACTACC CTCGTGGAAT CCTGACCCCA TGTAAATAAA ACCGTGACAG 8000

CTCATGGGGT GGGAGATATC GCTGTTCCTT AGGACCCTTT TACTAACCCT 8050

AATTCGATAG CATATGCTTC CCGTTGGGTA ACATATGCTA TTGAATTAGG 8100

GTTAGTCTGG ATAGTATATA CTACTACCCG GGAAGCATAT GCTACCCGTT 8150

TAGGGTTAAC AAGGGGGCCT TATAAACACT ATTGCTAATG CCCTCTTGAG 8200

GGTCCGCTTA TCGGTAGCTA CACAGGCCCC TCTGATTGAC GTTGGTGTAG 8250

CCTCCCGTAG TCTTCCTGGG CCCCTGGGAG GTACATGTCC CCCAGCATTG 8300

GTGTAAGAGC TTCAGCCAAG AGTTACACAT AAAGGCAATG TTGTGTTGCA 8350

GTCCACAGAC TGCAAAGTCT GCTCCAGGAT GAAAGCCACT CAGTGTTGGC 8400

AAATGTGCAC ATCCATTTAT AAGGATGTCA ACTACAGTCA GAGAACCCCT 8450

TTGTGTTTGG TCCCCCCCCG TGTCACATGT GGAACAGGGC CCAGTTGGCA 8500

AGTTGTACCA ACCAACTGAA GGGATTACAT GCACTGCCCC 8540

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 bases
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer UDEC690

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGCCTGCAGG ACATCCAGAT GACTCAGTCT 30

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 bases
(B) TYPE: Nucleic Acid

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(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer UDEC395

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCCAAGCTTA CTGGATGGTG GGAAGATGGA 30

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1M1 scFv (murine)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
ATG GCC GAC ATC CAG ATG ACT CAG TCT CCA                    30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

GCT TCA CTG TCT GCA TCT GTG GGA GAA ACT                    60
Ala Ser Leu Ser Ala Ser Val Gly Glu Thr
15                  20

GTC ACC ATC ACA TGT GGA GCA AGT GAG AAT                    90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
25                  30

ATT TAC GGT GCT TTA AAT TGG TAT CAG CGG                   120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Arg
35                  40

AAA CAG GGA AAA TCT CCT CAG CTC CTG ATC                   150
Lys Gln Gly Lys Ser Pro Gln Leu Leu Ile
45                  50

TAT GGT GCA ACC AAC TTG GCA GAT GGC ATG                   180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Met
55                  60

TCA TCG AGG TTC AGT GGC AGT GGA TCT GGT                   210
Ser Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

AGA CAG TAT TAT CTC AAG ATC AGT AGC CTG                   240
Arg Gln Tyr Tyr Leu Lys Ile Ser Ser Leu
75                  80

CAT CCT GAC GAT GTT GCA ACG TAT TAC TGT                   270
His Pro Asp Asp Val Ala Thr Tyr Tyr Cys
85                  90

CAA AAT GTG TTA AAT ACT CCT CTC ACG TTC                   300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100

GGT GCT GGG ACC AAG TTG GAG CTG AAA CGG                   330
Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg
105                 110

ACC GGA GGT GGC GGG TCG GGT GGC GGG GGA                   360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCG GGT GGC GGA GGG TCG CAG GTT CAG CTG                   390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130
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CAG CAG TCT GGA GCC GAG CTG ATG AAG CCT                          420
Gln Gln Ser Gly Ala Glu Leu Met Lys Pro
135                 140

GGG GCC TCA GTG AAG ATG TCC TGC AAG GCT                          450
Gly Ala Ser Val Lys Met Ser Cys Lys Ala
145                 150

ACT GGC TAC ATA TTC AGT AAC TAC TGG ATA                          480
Thr Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAG TGG ATA AAG CAG AGG CCT GGA CAT GGC                          510
Gln Trp Ile Lys Gln Arg Pro Gly His Gly
165                 170

CTT GAG TGG ATT GGT GAG ATT TTA CCT GGA                          540
Leu Glu Trp Ile Gly Glu Ile Leu Pro Gly
175                 180

AGT GGT TCT ACT GAG TAC ACT GAG AAC TTC                          570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
185                 190

AAG GAC AAG GCC GCA TTC ACT GCA GAT ACA                          600
Lys Asp Lys Ala Ala Phe Thr Ala Asp Thr
195                 200

TCC TCC AAC ACA GCC TAC ATG CAA CTC AGC                          630
Ser Ser Asn Thr Ala Tyr Met Gln Leu Ser
205                 210

AGC CTG ACA TCA GAG GAC TCT GCC GTC TAT                          660
Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr
215                 220

TAC TGT GCA AGA TAT TTC TTC GGT AGT AGC                          690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCC AAC TGG TAC TTC GAT GTC TGG GGC GCA                          720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Ala
235                 240

GGG ACC ACG GTC ACC GTC TCC TCA TGA                              747
Gly Thr Thr Val Thr Val Ser Ser
245
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 scFv CB (humanized)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                           30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                           60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                           90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CGT                          120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Arg
35                  40
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AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                          150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                          180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                          210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                          240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                          270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                          300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                          330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                          360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                          390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                          420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                          450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                          480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                          510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                          540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                          570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                          600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                          630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                          660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                          690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                          720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240
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```
GGA ACC CTG GTC ACT GTC TCG AGC TGA                                    747
Gly Thr Leu Val Thr Val Ser Ser
245

(2) INFORMATION FOR SEQ ID NO:9:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH:  726 base pairs
          (B) TYPE:  Nucleic Acid
          (C) STRANDEDNESS:  Double
          (D) TOPOLOGY:  linear

    (ii) MOLECULE TYPE:  Other nucleic acid
          (A) DESCRIPTION:5G1.1M1 VL HuK (chimeric light chain)

    (xi) SEQUENCE DESCRIPTION:  SEQ ID NO:9:

ATG GGA ATC CAA GGA GGG TCT GTC CTG TTC                                 30
Met Gly Ile Gln Gly Gly Ser Val Leu Phe
-25                 -20

GGG CTG CTG CTC GTC CTG GCT GTC TTC TGC                                 60
Gly Leu Leu Leu Val Leu Ala Val Phe Cys
-15                 -10

CAT TCA GGT CAT AGC CTG CAG GAC ATC CAG                                 90
His Ser Gly His Ser Leu Gln Asp Ile Gln
-5                   1                   5

ATG ACT CAG TCT CCA GCT TCA CTG TCT GCA                                 120
Met Thr Gln Ser Pro Ala Ser Leu Ser Ala
10                  15

TCT GTG GGA GAA ACT GTC ACC ATC ACA TGT                                 150
Ser Val Gly Glu Thr Val Thr Ile Thr Cys
20                  25

GGA GCA AGT GAG AAT ATT TAC GGT GCT TTA                                 180
Gly Ala Ser Glu Asn Ile Tyr Gly Ala Leu
30                  35

AAT TGG TAT CAG CGG AAA CAG GGA AAA TCT                                 210
Asn Trp Tyr Gln Arg Lys Gln Gly Lys Ser
40                  45

CCT CAG CTC CTG ATC TAT GGT GCA ACC AAC                                 240
Pro Gln Leu Leu Ile Tyr Gly Ala Thr Asn
50                  55

TTG GCA GAT GGC ATG TCA TCG AGG TTC AGT                                 270
Leu Ala Asp Gly Met Ser Ser Arg Phe Ser
60                  65

GGC AGT GGA TCT GGT AGA CAG TAT TAT CTC                                 300
Gly Ser Gly Ser Gly Arg Gln Tyr Tyr Leu
70                  75

AAG ATC AGT AGC CTG CAT CCT GAC GAT GTT                                 330
Lys Ile Ser Ser Leu His Pro Asp Asp Val
80                  85

GCA ACG TAT TAC TGT CAA AAT GTG TTA AAT                                 360
Ala Thr Tyr Tyr Cys Gln Asn Val Leu Asn
90                  95

ACT CCT CTC ACG TTC GGT GCT GGG ACC AAG                                 390
Thr Pro Leu Thr Phe Gly Ala Gly Thr Lys
100                 105

TTG GAG CTG AAA CGA ACT GTG GCT GCA CCA                                 420
Leu Glu Leu Lys Arg Thr Val Ala Ala Pro
110                 115

TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG                                 450
Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
120                 125
```

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```
CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG                    480
Gln Leu Lys Ser Gly Thr Ala Ser Val Val
130                 135

TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG                    510
Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu
140                 145

GCC AAA GTA CAG TGG AAG GTG GAT AAC GCC                    540
Ala Lys Val Gln Trp Lys Val Asp Asn Ala
150                 155

CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC                    570
Leu Gln Ser Gly Asn Ser Gln Glu Ser Val
160                 165

ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC                    600
Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
170                 175

AGC CTC AGC AGC ACC CTG ACG CTG AGC AAA                    630
Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys
180                 185

GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC                    660
Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
190                 195

TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG                    690
Cys Glu Val Thr His Gln Gly Leu Ser Ser
200                 205

CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG                    720
Pro Val Thr Lys Ser Phe Asn Arg Gly Glu
210                 215

TGT TAG                                                    726
Cys
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 750 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1M1 VH +HuG1 (chimeric Fd)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
ATG AAA TGG AGC TGG GTT ATT CTC TTC CTC                     30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
-15                 -10

CTG TCA GTA ACT GCA GGT GTC CAC TCC CAG                     60
Leu Ser Val Thr Ala Gly Val His Ser Gln
-5                  1

GTT CAG CTG CAG CAG TCT GGA GCT GAG CTG                     90
Val Gln Leu Gln Gln Ser Gly Ala Glu Leu
5                   10

ATG AAG CCT GGG GCC TCA GTG AAG ATG TCC                    120
Met Lys Pro Gly Ala Ser Val Lys Met Ser
15                  20

TGC AAG GCT ACT GGC TAC ATA TTC AGT AAC                    150
Cys Lys Ala Thr Gly Tyr Ile Phe Ser Asn
25                  30

TAC TGG ATA CAG TGG ATA AAG CAG AGG CCT                    180
Tyr Trp Ile Gln Trp Ile Lys Gln Arg Pro
35                  40

GGA CAT GGC CTT GAG TGG ATT GGT GAG ATT                    210
Gly His Gly Leu Glu Trp Ile Gly Glu Ile
45                  50
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TTA CCT GGA AGT GGT TCT ACT GAG TAC ACT                          240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Thr
55                  60

GAG AAC TTC AAG GAC AAG GCC GCA TTC ACT                          270
Glu Asn Phe Lys Asp Lys Ala Ala Phe Thr
65                  70

GCA GAT ACA TCC TCC AAC ACA GCC TAC ATG                          300
Ala Asp Thr Ser Ser Asn Thr Ala Tyr Met
75                  80

CAA CTC AGC AGC CTG ACA TCA GAG GAC TCT                          330
Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser
85                  90

GCC GTC TAT TAC TGT GCA AGA TAT TTC TTC                          360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
95                 100

GGT AGT AGC CCC AAC TGG TAC TTC GAT GTC                          390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
105                 110

TGG GGC GCA GGG ACC ACG GTC ACC GTC TCC                          420
Trp Gly Ala Gly Thr Thr Val Thr Val Ser
115                 120

TCA GCC TCC ACC AAG GGC CCA TCG GTC TTC                          450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT                          480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
135                 140

GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC                          510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG                          540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
155                 160

TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC                          570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC                          600
Val His Thr Phe Pro Ala Val Leu Gln Ser
175                 180

TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG                          630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG                          660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
195                 200

ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC                          690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG                          720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA                          750
Pro Lys Ser Cys Asp Lys Thr His Thr
225
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 750 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

-continued

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 VH + IGHRL (Humanized Fd)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
ATG AAG TGG AGC TGG GTT ATT CTC TTC CTC                    30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
-15                 -10

CTG TCA GTA ACT GCC GGC GTC CAC TCC CAA                    60
Leu Ser Val Thr Ala Gly Val His Ser Gln
-5                  1

GTC CAA CTG GTG CAA TCC GGC GCC GAG GTC                    90
Val Gln Leu Val Gln Ser Gly Ala Glu Val
5                   10

AAG AAG CCA GGG GCC TCA GTC AAA GTG TCC                    120
Lys Lys Pro Gly Ala Ser Val Lys Val Ser
15                  20

TGT AAA GCT AGC GGC TAT ATT TTT TCT AAT                    150
Cys Lys Ala Ser Gly Tyr Ile Phe Ser Asn
25                  30

TAT TGG ATT CAA TGG GTG CGT CAG GCC CCC                    180
Tyr Trp Ile Gln Trp Val Arg Gln Ala Pro
35                  40

GGG CAG GGC CTG GAA TGG ATG GGT GAG ATC                    210
Gly Gln Gly Leu Glu Trp Met Gly Glu Ile
45                  50

TTA CCG GGC TCT GGT AGC ACC GAA TAT GCC                    240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Ala
55                  60

CAA AAA TTC CAG GGC CGT GTT ACT ATG ACT                    270
Gln Lys Phe Gln Gly Arg Val Thr Met Thr
65                  70

GCG GAC ACT TCG ACT AGT ACA GCC TAC ATG                    300
Ala Asp Thr Ser Thr Ser Thr Ala Tyr Met
75                  80

GAG CTC TCC AGC CTG CGA TCG GAG GAC ACG                    330
Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr
85                  90

GCC GTC TAT TAT TGC GCG CGT TAT TTT TTT                    360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
95                 100

GGT TCT AGC CCG AAT TGG TAT TTT GAT GTT                    390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
105                 110

TGG GGT CAA GGA ACC CTG GTC ACT GTC TCG                    420
Trp Gly Gln Gly Thr Leu Val Thr Val Ser
115                 120

AGC GCC TCC ACC AAG GGC CCA TCG GTC TTC                    450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT                    480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
135                 140

GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC                    510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG                    540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
155                 160
```

-continued

```
TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC                          570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC                          600
Val His Thr Phe Pro Ala Val Leu Gln Ser
175                 180

TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG                          630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG                          660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
195                 200

ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC                          690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG                          720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA                          750
Pro Lys Ser Cys Asp Lys Thr His Thr
225                 230
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 750 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 VH + IGHRLC (Humanized Fd)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
ATG AAG TGG AGC TGG GTT ATT CTC TTC CTC                           30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
-15                 -10

CTG TCA GTA ACT GCC GGC GTC CAC TCC CAA                           60
Leu Ser Val Thr Ala Gly Val His Ser Gln
-5                   1

GTC CAA CTG GTG CAA TCC GGC GCC GAG GTC                           90
Val Gln Leu Val Gln Ser Gly Ala Glu Val
5                   10

AAG AAG CCA GGG GCC TCA GTC AAA GTG TCC                          120
Lys Lys Pro Gly Ala Ser Val Lys Val Ser
15                  20

TGT AAA GCT AGC GGC TAT ATT TTT TCT AAT                          150
Cys Lys Ala Ser Gly Tyr Ile Phe Ser Asn
25                  30

TAT TGG ATT CAA TGG GTG CGT CAG GCC CCC                          180
Tyr Trp Ile Gln Trp Val Arg Gln Ala Pro
35                  40

GGG CAG GGC CTG GAA TGG ATG GGT GAG ATC                          210
Gly Gln Gly Leu Glu Trp Met Gly Glu Ile
45                  50

TTA CCG GGC TCT GGT AGC ACC GAA TAT ACC                          240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Thr
55                  60

GAA AAT TTT AAA GAC CGT GTT ACT ATG ACG                          270
Glu Asn Phe Lys Asp Arg Val Thr Met Thr
65                  70
```

-continued

```
CGT GAC ACT TCG ACT AGT ACA GTA TAC ATG                    300
Arg Asp Thr Ser Thr Ser Thr Val Tyr Met
75                  80

GAG CTC TCC AGC CTG CGA TCG GAG GAC ACG                    330
Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr
85                  90

GCC GTC TAT TAT TGC GCG CGT TAT TTT TTT                    360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
95                 100

GGT TCT AGC CCG AAT TGG TAT TTT GAT GTT                    390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
105                 110

TGG GGT CAA GGA ACC CTG GTC ACT GTC TCG                    420
Trp Gly Gln Gly Thr Leu Val Thr Val Ser
115                 120

AGC GCC TCC ACC AAG GGC CCA TCG GTC TTC                    450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT                    480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
135                 140

GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC                    510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG                    540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
155                 160

TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC                    570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC                    600
Val His Thr Phe Pro Ala Val Leu Gln Ser
175                 180

TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG                    630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG                    660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
195                 200

ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC                    690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG                    720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA                    750
Pro Lys Ser Cys Asp Lys Thr His Thr
225                 230
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 726 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 VL +KLV56
(Humanized light chain)

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
ATG GGA ATC CAA GGA GGG TCT GTC CTG TTC                    30
Met Gly Ile Gln Gly Gly Ser Val Leu Phe
-25                 -20

GGG CTG CTG CTC GTC CTG GCT GTC TTC TGC                    60
Gly Leu Leu Leu Val Leu Ala Val Phe Cys
-15                 -10

CAT TCA GGT CAT AGC CTG CAG GAT ATC CAG                    90
His Ser Gly His Ser Leu Gln Asp Ile Gln
-5                   1                   5

ATG ACC CAG TCC CCG TCC TCC CTG TCC GCC                   120
Met Thr Gln Ser Pro Ser Ser Leu Ser Ala
10                  15

TCT GTG GGC GAT AGG GTC ACC ATC ACC TGC                   150
Ser Val Gly Asp Arg Val Thr Ile Thr Cys
20                  25

GGC GCC AGC GAA AAC ATC TAT GGC GCG CTG                   180
Gly Ala Ser Glu Asn Ile Tyr Gly Ala Leu
30                  35

AAC TGG TAT CAA CGT AAA CCT GGG AAA GCT                   210
Asn Trp Tyr Gln Arg Lys Pro Gly Lys Ala
40                  45

CCG AAG CTT CTG ATT TAC GGT GCG ACG AAC                   240
Pro Lys Leu Leu Ile Tyr Gly Ala Thr Asn
50                  55

CTG GCA GAT GGA GTC CCT TCT CGC TTC TCT                   270
Leu Ala Asp Gly Val Pro Ser Arg Phe Ser
60                  65

GGA TCC GGC TCC GGA ACG GAT TAC ACT CTG                   300
Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu
70                  75

ACC ATC AGC AGT CTG CAA CCT GAG GAC TTC                   330
Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe
80                  85

GCT ACG TAT TAC TGT CAG AAC GTT TTA AAT                   360
Ala Thr Tyr Tyr Cys Gln Asn Val Leu Asn
90                  95

ACT CCG TTG ACT TTC GGA CAG GGT ACC AAG                   390
Thr Pro Leu Thr Phe Gly Gln Gly Thr Lys
100                 105

GTG GAA ATA AAA CGA ACT GTG GCT GCA CCA                   420
Val Glu Ile Lys Arg Thr Val Ala Ala Pro
110                 115

TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG                   450
Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
120                 125

CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG                   480
Gln Leu Lys Ser Gly Thr Ala Ser Val Val
130                 135

TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG                   510
Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu
140                 145

GCC AAA GTA CAG TGG AAG GTG GAT AAC GCC                   540
Ala Lys Val Gln Trp Lys Val Asp Asn Ala
150                 155

CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC                   570
Leu Gln Ser Gly Asn Ser Gln Glu Ser Val
160                 165
```

-continued

```
ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC                          600
Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
170                 175

AGC CTC AGC AGC ACC CTG ACG CTG AGC AAA                          630
Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys
180                 185

GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC                          660
Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
190                 195

TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG                          690
Cys Glu Val Thr His Gln Gly Leu Ser Ser
200                 205

CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG                          720
Pro Val Thr Lys Ser Phe Asn Arg Gly Glu
210                 215

TGT TAG                                                          726
Cys
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 726 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 VL +KLV56B
(Humanized light chain)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
ATG GGA ATC CAA GGA GGG TCT GTC CTG TTC                           30
Met Gly Ile Gln Gly Gly Ser Val Leu Phe
-25                 -20

GGG CTG CTG CTC GTC CTG GCT GTC TTC TGC                           60
Gly Leu Leu Leu Val Leu Ala Val Phe Cys
-15                 -10

CAT TCA GGT CAT AGC CTG CAG GAT ATC CAG                           90
His Ser Gly His Ser Leu Gln Asp Ile Gln
-5                  1                5

ATG ACC CAG TCC CCG TCC TCC CTG TCC GCC                          120
Met Thr Gln Ser Pro Ser Ser Leu Ser Ala
10                  15

TCT GTG GGC GAT AGG GTC ACC ATC ACC TGC                          150
Ser Val Gly Asp Arg Val Thr Ile Thr Cys
20                  25

GGC GCC AGC GAA AAC ATC TAT GGC GCG CTG                          180
Gly Ala Ser Glu Asn Ile Tyr Gly Ala Leu
30                  35

AAC TGG TAT CAA CGT AAA CCT GGG AAA GCT                          210
Asn Trp Tyr Gln Arg Lys Pro Gly Lys Ala
40                  45

CCG AAG CTT CTG ATT TAC GGT GCG ACG AAC                          240
Pro Lys Leu Leu Ile Tyr Gly Ala Thr Asn
50                  55

CTG GCA GAT GGA GTC CCT TCT CGC TTC TCT                          270
Leu Ala Asp Gly Val Pro Ser Arg Phe Ser
60                  65

GGA TCC GGC TCC GGA ACG GAT TTC ACT CTG                          300
Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu
70                  75
```

-continued

```
ACC ATC AGC AGT CTG CAG CCT GAA GAC TTC                    330
Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe
80                  85

GCT ACG TAT TAC TGT CAG AAC GTT TTA AAT                    360
Ala Thr Tyr Tyr Cys Gln Asn Val Leu Asn
90                  95

ACT CCG TTG ACT TTC GGA CAG GGT ACC AAG                    390
Thr Pro Leu Thr Phe Gly Gln Gly Thr Lys
100                 105

GTG GAA ATA AAA CGA ACT GTG GCT GCA CCA                    420
Val Glu Ile Lys Arg Thr Val Ala Ala Pro
110                 115

TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG                    450
Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
120                 125

CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG                    480
Gln Leu Lys Ser Gly Thr Ala Ser Val Val
130                 135

TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG                    510
Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu
140                 145

GCC AAA GTA CAG TGG AAG GTG GAT AAC GCC                    540
Ala Lys Val Gln Trp Lys Val Asp Asn Ala
150                 155

CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC                    570
Leu Gln Ser Gly Asn Ser Gln Glu Ser Val
160                 165

ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC                    600
Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
170                 175

AGC CTC AGC AGC ACC CTG ACG CTG AGC AAA                    630
Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys
180                 185

GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC                    660
Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
190                 195

TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG                    690
Cys Glu Val Thr His Gln Gly Leu Ser Ser
200                 205

CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG                    720
Pro Val Thr Lys Ser Phe Asn Arg Gly Glu
210                 215

TGT TAG                                                    726
Cys
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 711 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 VL + O12
(Humanized light chain)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
ATG GAC ATG AGG GTC CCC GCT CAG CTC CTG                     30
Met Asp Met Arg Val Pro Ala Gln Leu Leu
-20                 -15
```

-continued

```
GGG CTC CTG CTA CTC TGG CTC CGA GGT GCC                    60
Gly Leu Leu Leu Leu Trp Leu Arg Gly Ala
-10                 -5

AGA TGT GAT ATC CAG ATG ACC CAG TCC CCG                    90
Arg Cys Asp Ile Gln Met Thr Gln Ser Pro
1               5

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                   120
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
10                  15

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                   150
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
20                  25

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                   180
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
30                  35

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                   210
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
40                  45

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                   240
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
50                  55

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                   270
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
60                  65

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                   300
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
70                  75

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                   330
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
80                  85

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                   360
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
90                  95

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGA                   390
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
100                 105

ACT GTG GCT GCA CCA TCT GTC TTC ATC TTC                   420
Thr Val Ala Ala Pro Ser Val Phe Ile Phe
110                 115

CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA                   450
Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
120                 125

ACT GCC TCT GTT GTG TGC CTG CTG AAT AAC                   480
Thr Ala Ser Val Val Cys Leu Leu Asn Asn
130                 135

TTC TAT CCC AGA GAG GCC AAA GTA CAG TGG                   510
Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp
140                 145

AAG GTG GAT AAC GCC CTC CAA TCG GGT AAC                   540
Lys Val Asp Asn Ala Leu Gln Ser Gly Asn
150                 155

TCC CAG GAG AGT GTC ACA GAG CAG GAC AGC                   570
Ser Gln Glu Ser Val Thr Glu Gln Asp Ser
160                 165

AAG GAC AGC ACC TAC AGC CTC AGC AGC ACC                   600
Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr
170                 175

CTG ACG CTG AGC AAA GCA GAC TAC GAG AAA                   630
Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys
180                 185
```

-continued

```
CAC AAA GTC TAC GCC TGC GAA GTC ACC CAT                          660
His Lys Val Tyr Ala Cys Glu Val Thr His
190                 195

CAG GGC CTG AGC TCG CCC GTC ACA AAG AGC                          690
Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
200                 205

TTC AAC AGG GGA GAG TGT TAG                                      711
Phe Asn Arg Gly Glu Cys
210
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 750 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 VH + IGHRLD
(Humanized Fd)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
ATG AAG TGG AGC TGG GTT ATT CTC TTC CTC                           30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
-15                 -10

CTG TCA GTA ACT GCC GGC GTC CAC TCC CAA                           60
Leu Ser Val Thr Ala Gly Val His Ser Gln
-5                  1

GTC CAA CTG GTG CAA TCC GGC GCC GAG GTC                           90
Val Gln Leu Val Gln Ser Gly Ala Glu Val
5                   10

AAG AAG CCA GGG GCC TCA GTC AAA GTG TCC                           120
Lys Lys Pro Gly Ala Ser Val Lys Val Ser
15                  20

TGT AAA GCT AGC GGC TAT ATT TTT TCT AAT                           150
Cys Lys Ala Ser Gly Tyr Ile Phe Ser Asn
25                  30

TAT TGG ATT CAA TGG GTG CGT CAG GCC CCC                           180
Tyr Trp Ile Gln Trp Val Arg Gln Ala Pro
35                  40

GGG CAG GGC CTG GAA TGG ATG GGT GAG ATC                           210
Gly Gln Gly Leu Glu Trp Met Gly Glu Ile
45                  50

TTA CCG GGC TCT GGT AGC ACC GAA TAT GCC                           240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Ala
55                  60

CAA AAA TTC CAG GGC CGT GTT ACT ATG ACT                           270
Gln Lys Phe Gln Gly Arg Val Thr Met Thr
65                  70

CGT GAC ACT TCG ACT AGT ACA GTA TAC ATG                           300
Arg Asp Thr Ser Thr Ser Thr Val Tyr Met
75                  80

GAG CTC TCC AGC CTG CGA TCG GAG GAC ACG                           330
Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr
85                  90

GCC GTC TAT TAT TGC GCG CGT TAT TTT TTT                           360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
95                 100

GGT TCT AGC CCG AAT TGG TAT TTT GAT GTT                           390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
105                 110
```

-continued

```
TGG GGT CAA GGA ACC CTG GTC ACT GTC TCG                                  420
Trp Gly Gln Gly Thr Leu Val Thr Val Ser
115                 120

AGC GCC TCC ACC AAG GGC CCA TCG GTC TTC                                  450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT                                  480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
135                 140

GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC                                  510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG                                  540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
155                 160

TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC                                  570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC                                  600
Val His Thr Phe Pro Ala Val Leu Gln Ser
175                 180

TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG                                  630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG                                  660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
195                 200

ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC                                  690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG                                  720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA                                  750
Pro Lys Ser Cys Asp Lys Thr His Thr
225                 230
```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 scFv DO12
(Humanized scFv)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                                   30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                                   60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                                   90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                                  120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
35                  40
```

-continued

```
AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                    150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                    180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                    210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                    240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                    270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                    300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT GCC CAA AAA TTC                    570
Ser Gly Ser Thr Glu Tyr Ala Gln Lys Phe
185                 190

CAG GGC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Gln Gly Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240
```

-continued

GGA ACC CTG GTC ACT GTC TCG AGC TGA 747
Gly Thr Leu Val Thr Val Ser Ser
245

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5248 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: Circular

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: pET Trc SO5/NI
prokaryotic expression vector

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TGGCGAATGG GACGCGCCCT GTAGCGGCGC ATTAAGCGCG GCGGGTGTGG 50

TGGTTACGCG CAGCGTGACC GCTACACTTG CCAGCGCCCT AGCGCCCGCT 100

CCTTTCGCTT TCTTCCCTTC CTTTCTCGCC ACGTTCGCCG GCTTTCCCCG 150

TCAAGCTCTA AATCGGGGGC TCCCTTTAGG GTTCCGATTT AGTGCTTTAC 200

GGCACCTCGA CCCCAAAAAA CTTGATTAGG GTGATGGTTC ACGTAGTGGG 250

CCATCGCCCT GATAGACGGT TTTTCGCCCT TTGACGTTGG AGTCCACGTT 300

CTTTAATAGT GGACTCTTGT TCCAAACTGG AACAACACTC AACCCTATCT 350

CGGTCTATTC TTTTGATTTA TAAGGGATTT TGCCGATTTC GGCCTATTGG 400

TTAAAAAATG AGCTGATTTA ACAAAAATTT AACGCGAATT TTAACAAAAT 450

ATTAACGTTT ACAATTTCAG GTGGCACTTT TCGGGGAAAT GTGCGCGGAA 500

CCCCTATTTG TTTATTTTTC TAAATACATT CAAATATGTA TCCGCTCATG 550

AGACAATAAC CCTGATAAAT GCTTCAATAA TATTGAAAAA GGAAGAGTAT 600

GAGTATTCAA CATTTCCGTG TCGCCCTTAT TCCCTTTTTT GCGGCATTTT 650

GCCTTCCTGT TTTTGCTCAC CCAGAAACGC TGGTGAAAGT AAAAGATGCT 700

GAAGATCAGT TGGGTGCACG AGTGGGTTAC ATCGAACTGG ATCTCAACAG 750

CGGTAAGATC CTTGAGAGTT TTCGCCCCGA AGAACGTTTT CCAATGATGA 800

GCACTTTTAA AGTTCTGCTA TGTGGCGCGG TATTATCCCG TATTGACGCC 850

GGGCAAGAGC AACTCGGTCG CCGCATACAC TATTCTCAGA ATGACTTGGT 900

TGAGTACTCA CCAGTCACAG AAAAGCATCT TACGGATGGC ATGACAGTAA 950

GAGAATTATG CAGTGCTGCC ATAACCATGA GTGATAACAC TGCGGCCAAC 1000

TTACTTCTGA CAACGATCGG AGGACCGAAG GAGCTAACCG CTTTTTTGCA 1050

CAACATGGGG GATCATGTAA CTCGCCTTGA TCGTTGGGAA CCGGAGCTGA 1100

ATGAAGCCAT ACCAAACGAC GAGCGTGACA CCACGATGCC TGCAGCAATG 1150

GCAACAACGT TGCGCAAACT ATTAACTGGC GAACTACTTA CTCTAGCTTC 1200

CCGGCAACAA TTAATAGACT GGATGGAGGC GGATAAAGTT GCAGGACCAC 1250

TTCTGCGCTC GGCCCTTCCG GCTGGCTGGT TTATTGCTGA TAAATCTGGA 1300

GCCGGTGAGC GTGGGTCTCG CGGTATCATT GCAGCACTGG GGCCAGATGG 1350

TAAGCCCTCC CGTATCGTAG TTATCTACAC GACGGGGAGT CAGGCAACTA 1400

TGGATGAACG AAATAGACAG ATCGCTGAGA TAGGTGCCTC ACTGATTAAG 1450

CATTGGTAAC TGTCAGACCA AGTTTACTCA TATATACTTT AGATTGATTT 1500

AAAACTTCAT TTTTAATTTA AAAGGATCTA GGTGAAGATC CTTTTTGATA 1550

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ATCTCATGAC CAAAATCCCT TAACGTGAGT TTTCGTTCCA CTGAGCGTCA	1600
GACCCCGTAG AAAAGATCAA AGGATCTTCT TGAGATCCTT TTTTTCTGCG	1650
CGTAATCTGC TGCTTGCAAA CAAAAAAACC ACCGCTACCA GCGGTGGTTT	1700
GTTTGCCGGA TCAAGAGCTA CCAACTCTTT TTCCGAAGGT AACTGGCTTC	1750
AGCAGAGCGC AGATACCAAA TACTGTCCTT CTAGTGTAGC CGTAGTTAGG	1800
CCACCACTTC AAGAACTCTG TAGCACCGCC TACATACCTC GCTCTGCTAA	1850
TCCTGTTACC AGTGGCTGCT GCCAGTGGCG ATAAGTCGTG TCTTACCGGG	1900
TTGGACTCAA GACGATAGTT ACCGGATAAG GCGCAGCGGT CGGGCTGAAC	1950
GGGGGGTTCG TGCACACAGC CCAGCTTGGA GCGAACGACC TACACCGAAC	2000
TGAGATACCT ACAGCGTGAG CTATGAGAAA GCGCCACGCT TCCCGAAGGG	2050
AGAAAGGCGG ACAGGTATCC GGTAAGCGGC AGGGTCGGAA CAGGAGAGCG	2100
CACGAGGGAG CTTCCAGGGG GAAACGCCTG GTATCTTTAT AGTCCTGTCG	2150
GGTTTCGCCA CCTCTGACTT GAGCGTCGAT TTTTGTGATG CTCGTCAGGG	2200
GGGCGGAGCC TATGGAAAAA CGCCAGCAAC GCGGCCTTTT TACGGTTCCT	2250
GGCCTTTTGC TGGCCTTTTG CTCACATGTT CTTTCCTGCG TTATCCCCTG	2300
ATTCTGTGGA TAACCGTATT ACCGCCTTTG AGTGAGCTGA TACCGCTCGC	2350
CGCAGCCGAA CGACCGAGCG CAGCGAGTCA GTGAGCGAGG AAGCGGAAGA	2400
GCGCCTGATG CGGTATTTTC TCCTTACGCA TCTGTGCGGT ATTTCACACC	2450
GCATATATGG TGCACTCTCA GTACAATCTG CTCTGATGCC GCATAGTTAA	2500
GCCAGTATAC ACTCCGCTAT CGCTACGTGA CTGGGTCATG GCTGCGCCCC	2550
GACACCCGCC AACACCCGCT GACGCGCCCT GACGGGCTTG TCTGCTCCCG	2600
GCATCCGCTT ACAGACAAGC TGTGACCGTC TCCGGGAGCT GCATGTGTCA	2650
GAGGTTTTCA CCGTCATCAC CGAAACGCGC GAGGCAGCTG CGGTAAAGCT	2700
CATCAGCGTG GTCGTGAAGC GATTCACAGA TGTCTGCCTG TTCATCCGCG	2750
TCCAGCTCGT TGAGTTTCTC CAGAAGCGTT AATGTCTGGC TTCTGATAAA	2800
GCGGGCCATG TTAAGGGCGG TTTTTTCCTG TTTGGTCACT GATGCCTCCG	2850
TGTAAGGGGG ATTTCTGTTC ATGGGGGTAA TGATACCGAT GAAACGAGAG	2900
AGGATGCTCA CGATACGGGT TACTGATGAT GAACATGCCC GGTTACTGGA	2950
ACGTTGTGAG GGTAAACAAC TGGCGGTATG GATGCGGCGG GACCAGAGAA	3000
AAATCACTCA GGGTCAATGC CAGCGCTTCG TTAATACAGA TGTAGGTGTT	3050
CCACAGGGTA GCCAGCAGCA TCCTGCGATG CAGATCCGGA ACATAATGGT	3100
GCAGGGCGCT GACTTCCGCG TTTCCAGACT TTACGAAACA CGGAAACCGA	3150
AGACCATTCA TGTTGTTGCT CAGGTCGCAG ACGTTTTGCA GCAGCAGTCG	3200
CTTCACGTTC GCTCGCGTAT CGGTGATTCA TTCTGCTAAC CAGTAAGGCA	3250
ACCCCGCCAG CCTAGCCGGG TCCTCAACGA CAGGAGCACG ATCATGCGCA	3300
CCCGTGGGGC CGCCATGCCG GCGATAATGG CCTGCTTCTC GCCGAAACGT	3350
TTGGTGGCGG GACCAGTGAC GAAGGCTTGA GCGAGGGCGT GCAAGATTCC	3400
GAATACCGCA AGCGACAGGC CGATCATCGT CGCGCTCCAG CGAAAGCGGT	3450
CCTCGCCGAA AATGACCCAG AGCGCTGCCG GCACCTGTCC TACGAGTTGC	3500
ATGATAAAGA AGACAGTCAT AAGTGCGGCG ACGATAGTCA TGCCCCGCGC	3550

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CCACCGGAAG GAGCTGACTG GGTTGAAGGC TCTCAAGGGC ATCGGTCGAG 3600

ATCCCGGTGC CTAATGAGTG AGCTAACTTA CATTAATTGC GTTGCGCTCA 3650

CTGCCCGCTT TCCAGTCGGG AAACCTGTCG TGCCAGCTGC ATTAATGAAT 3700

CGGCCAACGC GCGGGGAGAG GCGGTTTGCG TATTGGGCGC CAGGGTGGTT 3750

TTTCTTTTCA CCAGTGAGAC GGGCAACAGC TGATTGCCCT TCACCGCCTG 3800

GCCCTGAGAG AGTTGCAGCA AGCGGTCCAC GCTGGTTTGC CCCAGCAGGC 3850

GAAAATCCTG TTTGATGGTG GTTAACGGCG GGATATAACA TGAGCTGTCT 3900

TCGGTATCGT CGTATCCCAC TACCGAGATA TCCGCACCAA CGCGCAGCCC 3950

GGACTCGGTA ATGGCGCGCA TTGCGCCCAG CGCCATCTGA TCGTTGGCAA 4000

CCAGCATCGC AGTGGGAACG ATGCCCTCAT TCAGCATTTG CATGGTTTGT 4050

TGAAAACCGG ACATGGCACT CCAGTCGCCT TCCCGTTCCG CTATCGGCTG 4100

AATTTGATTG CGAGTGAGAT ATTTATGCCA GCCAGCCAGA CGCAGACGCG 4150

CCGAGACAGA ACTTAATGGG CCCGCTAACA GCGCGATTTG CTGGTGACCC 4200

AATGCGACCA GATGCTCCAC GCCCAGTCGC GTACCGTCTT CATGGGAGAA 4250

AATAATACTG TTGATGGGTG TCTGGTCAGA GACATCAAGA AATAACGCCG 4300

GAACATTAGT GCAGGCAGCT TCCACAGCAA TGGCATCCTG GTCATCCAGC 4350

GGATAGTTAA TGATCAGCCC ACTGACGCGT TGCGCGAGAA GATTGTGCAC 4400

CGCCGCTTTA CAGGCTTCGA CGCCGCTTCG TTCTACCATC GACACCACCA 4450

CGCTGGCACC CAGTTGATCG GCGCGAGATT TAATCGCCGC GACAATTTGC 4500

GACGGCGCGT GCAGGGCCAG ACTGGAGGTG GCAACGCCAA TCAGCAACGA 4550

CTGTTTGCCC GCCAGTTGTT GTGCCACGCG GTTGGGAATG TAATTCAGCT 4600

CCGCCATCGC CGCTTCCACT TTTTCCCGCG TTTTCGCAGA AACGTGGCTG 4650

GCCTGGTTCA CCACGCGGGA AACGGTCTGA TAAGAGACAC CGGCATACTC 4700

TGCGACATCG TATAACGTTA CTGGTTTCAC ATTCACCACC CTGAATTGAC 4750

TCTCTTCCGG GCGCTATCAT GCCATACCGC GAAAGGTTTT GCGCCATTCG 4800

ATGGTGTCCG GGATCTCGAC GCTCTCCCTT ATGCGACTCC TGCATTAGGA 4850

AGCAGCCCAG TAGTAGGTTG AGGCCGTTGA GCACCGCCGC CGCAAGGAAT 4900

GGTGCATGCG GTACCAGCTG TTGACAATTA ATCATCCGGC TCGTATAATA 4950

GTACTGTGTG GAATTGTGAG CGCTCACAAT TCCACACATC TAGAAATAAT 5000

TTTGTTTAAC TTTAAGAAGG AGATATACCA TGGAGATCTG GATCCATCGA 5050

TGAATTCGAG CTCCGTCGAC AAGCTTGCGG CCGCACTCGA GCACCACCAC 5100

CACCACCACT GAGATCCGGC TGCTAACAAA GCCCGAAAGG AAGCTGAGTT 5150

GGCTGCTGCC ACCGCTGAGC AATAACTAGC ATAACCCCTT GGGGCCTCTA 5200

AACGGGTCTT GAGGGGTTTT TTGCTGAAAG GAGGAACTAT ATCCGGAT 5248

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 783 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: N19/8 scFv (His Tagged)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```
ATG GCC AAT ATT GTG CTG ACC CAA TCT CCA                                  30
Met Ala Asn Ile Val Leu Thr Gln Ser Pro
1               5                   10

GCT TCT TTG GCT GTG TCT CTA GGG CAG AGG                                  60
Ala Ser Leu Ala Val Ser Leu Gly Gln Arg
15                  20

GCC ACC ATA TCC TGC AGA GCC AGT GAA AGT                                  90
Ala Thr Ile Ser Cys Arg Ala Ser Glu Ser
25                  30

GTT GAT AGT TAT GAC AAT AGT TTT ATG CAC                                 120
Val Asp Ser Tyr Asp Asn Ser Phe Met His
35                  40

TGG TAC CAG CAG AAA CCA GGA CAG CCA CCC                                 150
Trp Tyr Gln Gln Lys Pro Gly Gln Pro Pro
45                  50

AAA CTC CTC ATC TTT CTT GCA TCC AAC CTA                                 180
Lys Leu Leu Ile Phe Leu Ala Ser Asn Leu
55                  60

GAA TCT GGG GTC CCT GCC AGG TTC AGT GGC                                 210
Glu Ser Gly Val Pro Ala Arg Phe Ser Gly
65                  70

AGT GGG TCT AGG ACA GAC TTC ACC CTC ACC                                 240
Ser Gly Ser Arg Thr Asp Phe Thr Leu Thr
75                  80

ATT GAT CCT GTG GAG GCT GAT GAT GCT GCA                                 270
Ile Asp Pro Val Glu Ala Asp Asp Ala Ala
85                  90

ACC TAT TAC TGT CAG CAA AAT AAT GAG GTT                                 300
Thr Tyr Tyr Cys Gln Gln Asn Asn Glu Val
95                 100

CCG AAC ACG TTC GGA GGG GGG ACC AAG CTG                                 330
Pro Asn Thr Phe Gly Gly Gly Thr Lys Leu
105                 110

GAA ATA AAA CGG ACC GGA GGT GGC GGG TCG                                 360
Glu Ile Lys Arg Thr Gly Gly Gly Gly Ser
115                 120

GGT GGC GGG GGA TCG GGT GGC GGA GGG TCG                                 390
Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
125                 130

GAC GTC AAG CTC GTG GAG TCT GGG GGA GAC                                 420
Asp Val Lys Leu Val Glu Ser Gly Gly Asp
135                 140

TTA GTG AAG CTT GGA GGG TCC CTG AAA CTC                                 450
Leu Val Lys Leu Gly Gly Ser Leu Lys Leu
145                 150

TCC TGT GCA GCC TCT GGA TTC ACC TTC AGT                                 480
Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
155                 160

AGC TAT TAT ATG TCT TGG GTT CGC CAG ATT                                 510
Ser Tyr Tyr Met Ser Trp Val Arg Gln Ile
165                 170

TCA GAG AAG AGG CTG GAG TTG GTC GCA GCC                                 540
Ser Glu Lys Arg Leu Glu Leu Val Ala Ala
175                 180

ATT AAT AGT AAT GGT GAT AGC ACC TAC TAT                                 570
Ile Asn Ser Asn Gly Asp Ser Thr Tyr Tyr
185                 190
```

-continued

```
CCA GAC ACT GTG AAG GGC CGA TTC ACC ATC                          600
Pro Asp Thr Val Lys Gly Arg Phe Thr Ile
195                 200

TCC AGA GAC AAT GCC AAG AGC ACC CTG GAT                          630
Ser Arg Asp Asn Ala Lys Ser Thr Leu Asp
205                 210

CTG CAA ATG AGC AGT CTG AAG TCT GAG GAC                          660
Leu Gln Met Ser Ser Leu Lys Ser Glu Asp
215                 220

ACA GCC TTG TAT TTC TGT GTA AGA GAG ACT                          690
Thr Ala Leu Tyr Phe Cys Val Arg Glu Thr
225                 230

TAT TAC TAC GGG ATT AGT CCC GTC TTC GAT                          720
Tyr Tyr Tyr Gly Ile Ser Pro Val Phe Asp
235                 240

GTC TGG GGC ACA GGG ACC ACG GTC ACC GTC                          750
Val Trp Gly Thr Gly Thr Thr Val Thr Val
245                 250

TCC TCA CTC GAG CAC CAC CAC CAC CAC CAC                          780
Ser Ser Leu Glu His His His His His His
255                 260

TGA                                                              783
```

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 scFv C012 (humanized)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                           30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                           60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                           90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                          120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
35                  40

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                          150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                          180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                          210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                          240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                          270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90
```

-continued

```
CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                    300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                  100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                    570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                        747
Gly Thr Leu Val Thr Val Ser Ser
245
```

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 scFv DO12B
(Humanized scFv)

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                                  30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                                  60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC CGT GCT AGC GAA AAC                                  90
Val Thr Ile Thr Cys Arg Ala Ser Glu Asn
25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                                 120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
35                  40

AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                                 150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                                 180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                                 210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                                 240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                                 270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                                 300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                                 330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                                 360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                                 390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                                 420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                                 450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                                 480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                                 510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                                 540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT GCC CAA AAA TTC                                 570
Ser Gly Ser Thr Glu Tyr Ala Gln Lys Phe
185                 190
```

-continued

```
CAG GGC CGT GTT ACT ATG ACG CGT GAC ACT                          600
Gln Gly Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                          630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                          660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                          690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                          720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                              747
Gly Thr Leu Val Thr Val Ser Ser
245
```

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 scFv DO12C
(Humanized scFv)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                           30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                           60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                           90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                          120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
35                  40

AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                          150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AGC CTG CAG TCT GGA GTC                          180
Tyr Gly Ala Thr Ser Leu Gln Ser Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                          210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                          240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                          270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                          300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100
```

-continued

```
GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT GCC CAA AAA TTC                    570
Ser Gly Ser Thr Glu Tyr Ala Gln Lys Phe
185                 190

CAG GGC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Gln Gly Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                        747
Gly Thr Leu Val Thr Val Ser Ser
245
```

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 scFv DO12D
(Humanized scFv)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                     30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10
```

-continued

```
TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                    60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC CGT GCT AGC GAA AAC                    90
Val Thr Ile Thr Cys Arg Ala Ser Glu Asn
25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                   120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
35                  40

AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                   150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AGC CTG CAG TCT GGA GTC                   180
Tyr Gly Ala Thr Ser Leu Gln Ser Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                   210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                   240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                   270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                   300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                   330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                   360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                   390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                   420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                   450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                   480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                   510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                   540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT GCC CAA AAA TTC                   570
Ser Gly Ser Thr Glu Tyr Ala Gln Lys Phe
185                 190

CAG GGC CGT GTT ACT ATG ACG CGT GAC ACT                   600
Gln Gly Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                   630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210
```

-continued

```
AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                                  660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                                  690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                                  720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                                      747
Gly Thr Leu Val Thr Val Ser Ser
245
```

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 scFv C013 (humanized)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                                   30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                                   60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC CGT GCT AGC GAA AAC                                   90
Val Thr Ile Thr Cys Arg Ala Ser Glu Asn
25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                                  120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
35                  40

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                                  150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                                  180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                                  210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                                  240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                                  270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                                  300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                                  330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                                  360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120
```

-continued

```
TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                                  390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                                  420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                                  450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                                  480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                                  510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                                  540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                                  570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                                  600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                                  630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                                  660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                                  690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                                  720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                                      747
Gly Thr Leu Val Thr Val Ser Ser
245
```

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 scFv C014 (humanized)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                                   30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                                   60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                                   90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
25                  30
```

-continued

```
ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                    120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
35                  40

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                    150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AGC CTG CAG TCT GGA GTC                    180
Tyr Gly Ala Thr Ser Leu Gln Ser Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                    210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                    240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                    270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                    300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                    570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230
```

-continued

```
CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                          720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                             747
Gly Thr Leu Val Thr Val Ser Ser
245
```

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 scFv C015 (humanized)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                          30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                          60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC CGT GCT AGC GAA AAC                          90
Val Thr Ile Thr Cys Arg Ala Ser Glu Asn
25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                         120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
35                  40

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                         150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AGC CTG CAG TCT GGA GTC                         180
Tyr Gly Ala Thr Ser Leu Gln Ser Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                         210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                         240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                         270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                         300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                         330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                         360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                         390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                         420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140
```

-continued

```
GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                    570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                        747
Gly Thr Leu Val Thr Val Ser Ser
245
```

What is claimed is:

1. An antibody comprising at least one antibody-antigen binding site, said antibody exhibiting specific binding to human complement component C5, said specific binding being targeted to the alpha chain of human complement component C5, wherein the antibody 1) inhibits complement activation in a human body fluid, 2) inhibits the binding of purified human complement component C5 to either human complement component C3 or human complement component C4, and 3) does not specifically bind to the human complement activation product free C5a.

2. The antibody of claim 1 wherein the inhibition of complement activation in the human body fluid is measurable as an increment of blockade of C5a generation and an increment of blockade of complement hemolytic activity in the body fluid, said increment of blockade of C5a generation being substantially equal to said increment of blockade of complement hemolytic activity.

3. The antibody of claim 1 wherein, upon binding to human C5, there is a 60% to 90% reduction in the ability of C5 to bind to human complement component C3.

4. The antibody of claim 1 wherein, upon binding to human C5, there is a 60% to 90% reduction in the ability of C5 to bind to human complement component C4.

5. The antibody of claim 1 wherein the antibody binds specifically to an isolated oligopeptide comprising an amino acid sequence corresponding to amino acid 8 through amino acid 12 of SEQ ID NO:1.

6. The antibody of claim 1 wherein the inhibition of complement activation in the human body fluid is measurable as a substantially complete blockade of C5a generation in the body fluid and a substantially complete blockade of complement hemolytic activity in the body fluid when the antibody is added to the body fluid at a concentration yielding a ratio equal to or less than 10 moles of antibody-antigen binding sites of the antibody to 1 mole of human C5 in the body fluid.

7. The antibody of claim 1 wherein the antibody is a humanized antibody.

8. The antibody of claim 1 wherein the antibody is an scFv.

9. The antibody of claim 1, wherein, when administered to a human patient via intravenous infusion, the antibody provides complete complement inhibition at dosages below 0.005 g/kg.

10. The antibody of claim 1, wherein, when administered to a human patient via intravenous infusion, the antibody provides therapeutic benefits at dosages below 0.0022 g/kg.

11. The antibody of claim 10, wherein the antibody is administered in association with an extracorporeal circulation procedure.

12. The antibody of claim 1 wherein the inhibition of complement activation in the human body fluid is measurable as a substantially complete blockade of C5a generation in the body fluid and a substantially complete blockade of complement hemolytic activity in the body fluid when the

antibody is added to the body fluid at a concentration yielding a ratio equal to or less than 3 moles of antibody-antigen binding sites of the antibody to 1 mole of human C5 in the body fluid.

13. The antibody of claim 1, wherein, when administered to a human patient via intravenous infusion, the antibody provides therapeutically effective complement inhibition at dosages below 0.003 g/kg.

14. A sterile non-pyrogenic therapeutic agent comprising the antibody of claim 1 in a formulation suitable for administration to a human.

15. The therapeutic agent of claim 14 wherein the antibody is a humanized immunoglobulin.

16. The therapeutic agent of claim 14 wherein the antibody is an scFv.

17. The therapeutic agent of claim 14 wherein the antibody is made up of two or more heterodimeric subunits each containing one heavy and one light chain.

18. Antibody 5G1.1 scFv CB (humanized) having the amino acid sequence encoded by the nucleic acid sequence of SEQ ID NO:8.

19. An isolated antigen binding protein comprising:

1) a variable light region CDR1 comprising an amino acid sequence corresponding to amino acid residues 26–36 of SEQ ID NO:8,

2) a variable light region CDR2 comprising an amino acid sequence corresponding to amino acid residues 52–58 of SEQ ID NO:8,

3) a variable light region CDR3 comprising an amino acid sequence corresponding to amino acid residues 91 through amino acid 99 of SEQ ID NO:8,

4) a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid residues 152 through amino acid 161 of SEQ ID NO:8,

5) a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid residues 176 through amino acid 192 of SEQ ID NO:8,

6) a variable heavy region CDR3 comprising an amino acid sequence corresponding to amino acid residues 225 through amino acid 237 of SEQ ID NO:8,

said protein exhibiting specific binding to human complement component C5, said specific binding being targeted to the alpha chain of human complement component C5, wherein the protein inhibits complement activation in a human body fluid and does not specifically bind to the human complement activation product free C5a.

20. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO: 12.

21. Hybridoma 5G1.1 having ATCC designation HB-11625.

22. An antibody produced by the hybridoma of claim 21.

23. An antibody comprising at least one antibody-antigen binding site, said antibody exhibiting specific binding to human complement component C5, said specific binding being targeted to the alpha chain of human complement component C5, wherein:

(A) the antibody inhibits (i) C5b-9-mediated hemolysis and (ii) C5a generation in a fluid comprising human serum; and

(B) the antibody does not specifically bind to the human complement activation product free C5a.

* * * * *

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ROCKVILLE MD 20850

MAINTENANCE FEE STATEMENT

According to the records of the U.S.Patent and Trademark Office (USPTO), the maintenance fee and any necessary surcharge have been timely paid for the patent listed below. The "PYMT DATE" column indicates the payment date (i.e., the date the payment was filed).

The payment shown below is subject to actual collection. If the payment is refused or charged back by a financial institution, the payment will be void and the maintenance fee and any necessary surcharge unpaid.

Direct any questions about this statement to: Mail Stop M Correspondence, Director of the USPTO, P.O.Box 1450, Alexandria, VA 22313-1450.

PATENT NUMBER	FEE AMT	SUR CHARGE	PYMT DATE	U.S. APPLICATION NUMBER	PATENT ISSUE DATE	APPL. FILING DATE	PAYMENT YEAR	SMALL ENTITY?	ATTY DKT NUMBER
6,355,245	**$900.00**	**$0.00**	**09/12/05**	**08/487,283**	**03/12/02**	**06/07/95**	**04**	**NO**	**ALX-152.1CIP**

PTOL-439 (Rev. 09/2006)

Exhibit D

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page, and Column 1, lines 1-3,
Delete the title "**C5-SPECIFIC ANTIBODIES FOR THE TREATMENT OF INFLAMMATORY DISEASES**" and insert therefor -- **ANTIBODIES TO HUMAN COMPLEMENT COMPONENT C5** --.

Column 19,
Line 54, delete "(SEQ ID NO:1)".
Line 55, following "KSSKC peptide", insert -- (SEQ ID NO:1) --.

Column 55,
Please delete lines 50-53, and insert therefor:

```
Val Ile Asp His Gln Gly Thr Lys Ser Ser
                 5                  10

Lys Cys Val Arg Gln Lys Val Glu Gly Ser Ser
                15                  20
```

Column 57,
Please delete lines 22-37, and insert therefor:

```
                  Met Gly Leu Leu Gly Ile Leu Cys Phe Leu
                             -15                 -10

 Ile Phe Leu Gly Lys Thr Trp Gly Gln Glu Gln Thr Tyr Val
            -5              -1                   5

 Ile Ser Ala Pro Lys Ile Phe Arg Val Gly Ala Ser Glu Asn
           10                  15                  20

 Ile Val Ile Gln Val Tyr Gly Tyr Thr Glu Ala Phe Asp Ala
             25                  30

 Thr Ile Ser Ile Lys Ser Tyr Pro Asp Lys Lys Phe Ser Tyr
  35                 40                  45

 Ser Ser Gly His Val His Leu Ser Ser Glu Asn Lys Phe Gln
      50                 55                  60

 Asn Ser Ala Ile Leu Thr Ile Gln Pro Lys Gln Leu Pro Gly
         65                  70                  75

 Gly Gln Asn Pro Val Ser Tyr Val Tyr Leu Glu Val Val Ser
           80                  85                  90
```

Exhibit E

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 57,
Please delete lines 38-55, and insert therefor:

```
Lys His Phe Ser Lys Ser Lys Arg Met Pro Ile Thr Tyr Asp
                95                  100

Asn Gly Phe Leu Phe Ile His Thr Asp Lys Pro Val Tyr Thr
105                 110                 115

Pro Asp Gln Ser Val Lys Val Arg Val Tyr Ser Leu Asn Asp
    120                 125                 130

Asp Leu Lys Pro Ala Lys Arg Glu Thr Val Leu Thr Phe Ile
        135                 140                 145

Asp Pro Glu Gly Ser Glu Val Asp Met Val Glu Glu Ile Asp
            150                 155                 160

His Ile Gly Ile Ile Ser Phe Pro Asp Phe Lys Ile Pro Ser
                165                 170

Asn Pro Arg Tyr Gly Met Trp Thr Ile Lys Ala Lys Tyr Lys
175                 180                 185

Glu Asp Phe Ser Thr Thr Gly Thr Ala Tyr Phe Glu Val Lys
    190                 195                 200

Glu Tyr Val Leu Pro His Phe Ser Val Ser Ile Glu Pro Glu
        205                 210                 215
```

Column 59,
Please delete lines 1-4, and insert therefor:

```
Tyr Asn Phe Ile Gly Tyr Lys Asn Phe Lys Asn Phe Glu Ile
            220                 225                 230

Thr Ile Lys Ala Arg Tyr Phe Tyr Asn Lys Val Val Thr Glu
                235                 240
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 59,

Please delete lines 5-28, and insert therefor:

```
Ala Asp Val Tyr Ile Thr Phe Gly Ile Arg Glu Asp Leu Lys
245                 250                 255

Asp Asp Gln Lys Glu Met Met Gln Thr Ala Met Gln Asn Thr
    260                 265                 270

Met Leu Ile Asn Gly Ile Ala Gln Val Thr Phe Asp Ser Glu
        275                 280                 285

Thr Ala Val Lys Glu Leu Ser Tyr Tyr Ser Leu Glu Asp Leu
            290                 295                 300

Asn Asn Lys Tyr Leu Tyr Ile Ala Val Thr Val Ile Glu Ser
                305                 310

Thr Gly Gly Phe Ser Glu Glu Ala Glu Ile Pro Gly Ile Lys
315                 320                 325

Tyr Val Leu Ser Pro Tyr Lys Leu Asn Leu Val Ala Thr Pro
    330                 335                 340

Leu Phe Leu Lys Pro Gly Ile Pro Tyr Pro Ile Lys Val Gln
        345                 350                 355

Val Lys Asp Ser Leu Asp Gln Leu Val Gly Gly Val Pro Val
            360                 365                 370

Ile Leu Asn Ala Gln Thr Ile Asp Val Asn Gln Glu Thr Ser
                375                 380

Asp Leu Asp Pro Ser Lys Ser Val Thr Arg Val Asp Asp Gly
385                 390                 395

Val Ala Ser Phe Val Leu Asn Leu Pro Ser Gly Val Thr Val
    400                 405                 410
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 59,

Please delete lines 29-52, and insert therefor:

```
Leu Glu Phe Asn Val Lys Thr Asp Ala Pro Asp Leu Pro Glu
        415                 420                 425

Glu Asn Gln Ala Arg Glu Gly Tyr Arg Ala Ile Ala Tyr Ser
            430                 435                 440

Ser Leu Ser Gln Ser Tyr Leu Tyr Ile Asp Trp Thr Asp Asn
                445                 450

His Lys Ala Leu Leu Val Gly Glu His Leu Asn Ile Ile Val
455                 460                 465

Thr Pro Lys Ser Pro Tyr Ile Asp Lys Ile Thr His Tyr Asn
    470                 475                 480

Tyr Leu Ile Leu Ser Lys Gly Lys Ile Ile His Phe Gly Thr
        485                 490                 495

Arg Glu Lys Phe Ser Asp Ala Ser Tyr Gln Ser Ile Asn Ile
            500                 505                 510

Pro Val Thr Gln Asn Met Val Pro Ser Ser Arg Leu Leu Val
                515                 520

Tyr Tyr Ile Val Thr Gly Glu Gln Thr Ala Glu Leu Val Ser
525                 530                 535

Asp Ser Val Trp Leu Asn Ile Glu Glu Lys Cys Gly Asn Gln
    540                 545                 550

Leu Gln Val His Leu Ser Pro Asp Ala Asp Ala Tyr Ser Pro
        555                 560                 565

Gly Gln Thr Val Ser Leu Asn Met Ala Thr Gly Met Asp Ser
            570                 575                 580
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 61,

Please delete lines 1-26, and insert therefor:

```
Trp Val Ala Leu Ala Ala Val Asp Ser Ala Val Tyr Gly Val
                585                 590

Gln Arg Gly Ala Lys Lys Pro Leu Glu Arg Val Phe Gln Phe
595                 600                 605

Leu Glu Lys Ser Asp Leu Gly Cys Gly Ala Gly Gly Gly Leu
    610                 615                 620

Asn Asn Ala Asn Val Phe His Leu Ala Gly Leu Thr Phe Leu
        625                 630                 635

Thr Asn Ala Asn Ala Asp Asp Ser Gln Glu Asn Asp Glu Pro
            640                 645                 650

Cys Lys Glu Ile Leu Arg Pro Arg Arg Thr Leu Gln Lys Lys
                655                 660

Ile Glu Glu Ile Ala Ala Lys Tyr Lys His Ser Val Val Lys
665                 670                 675

Lys Cys Cys Tyr Asp Gly Ala Cys Val Asn Asn Asp Glu Thr
    680                 685                 690

Cys Glu Gln Arg Ala Ala Arg Ile Ser Leu Gly Pro Arg Cys
        695                 700                 705

Ile Lys Ala Phe Thr Glu Cys Cys Val Val Ala Ser Gln Leu
            710                 715                 720

Arg Ala Asn Ile Ser His Lys Asp Met Gln Leu Gly Arg Leu
                725                 730

His Met Lys Thr Leu Leu Pro Val Ser Lys Pro Glu Ile Arg
735                 740                 745

Ser Tyr Phe Pro Glu Ser Trp Leu Trp Glu Val His Leu Val
    750                 755                 760
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 61,
Please delete lines 27-52, and insert therefor:

```
Pro Arg Arg Lys Gln Leu Gln Phe Ala Leu Pro Asp Ser Leu
        765                 770                 775

Thr Thr Trp Glu Ile Gln Gly Ile Gly Ile Ser Asn Thr Gly
            780                 785                 790

Ile Cys Val Ala Asp Thr Val Lys Ala Lys Val Phe Lys Asp
                795                 800

Val Phe Leu Glu Met Asn Ile Pro Tyr Ser Val Val Arg Gly
805                 810                 815

Glu Gln Ile Gln Leu Lys Gly Thr Val Tyr Asn Tyr Arg Thr
    820                 825                 830

Ser Gly Met Gln Phe Cys Val Lys Met Ser Ala Val Glu Gly
        835                 840                 845

Ile Cys Thr Ser Glu Ser Pro Val Ile Asp His Gln Gly Thr
            850                 855                 860

Lys Ser Ser Lys Cys Val Arg Gln Lys Val Glu Gly Ser Ser
                865                 870

Ser His Leu Val Thr Phe Thr Val Leu Pro Leu Glu Ile Gly
875                 880                 885

Leu His Asn Ile Asn Phe Ser Leu Glu Thr Trp Phe Gly Lys
    890                 895                 900

Glu Ile Leu Val Lys Thr Leu Arg Val Val Pro Glu Gly Val
        905                 910                 915

Lys Arg Glu Ser Tyr Ser Gly Val Thr Leu Asp Pro Arg Gly
            920                 925                 930

Ile Tyr Gly Thr Ile Ser Arg Arg Lys Glu Phe Pro Tyr Arg
                935                 940
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 63,
Please delete lines 1-26, and insert therefor:

```
Ile Pro Leu Asp Leu Val Pro Lys Thr Glu Ile Lys Arg Ile
945                 950                 955

Leu Ser Val Lys Gly Leu Leu Val Gly Glu Ile Leu Ser Ala
    960                 965                 970

Val Leu Ser Gln Glu Gly Ile Asn Ile Leu Thr His Leu Pro
        975                 980                 985

Lys Gly Ser Ala Glu Ala Glu Leu Met Ser Val Val Pro Val
            990                 995                 1000

Phe Tyr Val Phe His Tyr Leu Glu Thr Gly Asn His Trp Asn
                1005                1010

Ile Phe His Ser Asp Pro Leu Ile Glu Lys Gln Lys Leu Lys
1015                1020                1025

Lys Lys Leu Lys Glu Gly Met Leu Ser Ile Met Ser Tyr Arg
    1030                1035                1040

Asn Ala Asp Tyr Ser Tyr Ser Val Trp Lys Gly Gly Ser Ala
        1045                1050                1055

Ser Thr Trp Leu Thr Ala Phe Ala Leu Arg Val Leu Gly Gln
            1060                1065                1070

Val Asn Lys Tyr Val Glu Gln Asn Gln Asn Ser Ile Cys Asn
                1075                1080

Ser Leu Leu Trp Leu Val Glu Asn Tyr Gln Leu Asp Asn Gly
1085                1090                1095

Ser Phe Lys Glu Asn Ser Gln Tyr Gln Pro Ile Lys Leu Gln
    1100                1105                1110

Gly Thr Leu Pro Val Glu Ala Arg Glu Asn Ser Leu Tyr Leu
        1115                1120                1125
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 63,
Please delete lines 27-52, and insert therefor:

```
Thr Ala Phe Thr Val Ile Gly Ile Arg Lys Ala Phe Asp Ile
            1130                1135                1140

Cys Pro Leu Val Lys Ile Asp Thr Ala Leu Ile Lys Ala Asp
                1145                1150

Asn Phe Leu Leu Glu Asn Thr Leu Pro Ala Gln Ser Thr Phe
1155                1160                1165

Thr Leu Ala Ile Ser Ala Tyr Ala Leu Ser Leu Gly Asp Lys
    1170                1175                1180

Thr His Pro Gln Phe Arg Ser Ile Val Ser Ala Leu Lys Arg
        1185                1190                1195

Glu Ala Leu Val Lys Gly Asn Pro Pro Ile Tyr Arg Phe Trp
            1200                1205                1210

Lys Asp Asn Leu Gln His Lys Asp Ser Ser Val Pro Asn Thr
                1215                1220

Gly Thr Ala Arg Met Val Glu Thr Thr Ala Tyr Ala Leu Leu
1225                1230                1235

Thr Ser Leu Asn Leu Lys Asp Ile Asn Tyr Val Asn Pro Val
    1240                1245                1250

Ile Lys Trp Leu Ser Glu Glu Gln Arg Tyr Gly Gly Gly Phe
        1255                1260                1265

Tyr Ser Thr Gln Asp Thr Ile Asn Ala Ile Glu Gly Leu Thr
            1270                1275                1280

Glu Tyr Ser Leu Leu Val Lys Gln Leu Arg Leu Ser Met Asp
                1285                1290

Ile Asp Val Ser Tyr Lys His Lys Gly Ala Leu His Asn Tyr
1295                1300                1305
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 65,

Please delete lines 1-26, and insert therefor:

```
Lys Met Thr Asp Lys Asn Phe Leu Gly Arg Pro Val Glu Val
    1310                1315                1320

Leu Leu Asn Asp Asp Leu Ile Val Ser Thr Gly Phe Gly Ser
        1325                1330                1335

Gly Leu Ala Thr Val His Val Thr Thr Val Val His Lys Thr
            1340                1345                1350

Ser Thr Ser Glu Glu Val Cys Ser Phe Tyr Leu Lys Ile Asp
                1355                1360

Thr Gln Asp Ile Glu Ala Ser His Tyr Arg Gly Tyr Gly Asn
1365                1370                1375

Ser Asp Tyr Lys Arg Ile Val Ala Cys Ala Ser Tyr Lys Pro
    1380                1385                1390

Ser Arg Glu Glu Ser Ser Ser Gly Ser Ser His Ala Val Met
        1395                1400                1405

Asp Ile Ser Leu Pro Thr Gly Ile Ser Ala Asn Glu Glu Asp
            1410                1415                1420

Leu Lys Ala Leu Val Glu Gly Val Asp Gln Leu Phe Thr Asp
                1425                1430

Tyr Gln Ile Lys Asp Gly His Val Ile Leu Gln Leu Asn Ser
1435                1440                1445

Ile Pro Ser Ser Asp Phe Leu Cys Val Arg Phe Arg Ile Phe
    1450                1455                1460

Glu Leu Phe Glu Val Gly Phe Leu Ser Pro Ala Thr Phe Thr
        1465                1470                1475

Val Tyr Glu Tyr His Arg Pro Asp Lys Gln Cys Thr Met Phe
            1480                1485                1490
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 65,
Please delete lines 27-50, and insert therefor:

```
Tyr Ser Thr Ser Asn Ile Lys Ile Gln Lys Val Cys Glu Gly
                1495                1500

Ala Ala Cys Lys Cys Val Glu Ala Asp Cys Gly Gln Met Gln
1505                1510                1515

Glu Glu Leu Asp Leu Thr Ile Ser Ala Glu Thr Arg Lys Gln
    1520                1525                1530

Thr Ala Cys Lys Pro Glu Ile Ala Tyr Ala Tyr Lys Val Ser
        1535                1540                1545

Ile Thr Ser Ile Thr Val Glu Asn Val Phe Val Lys Tyr Lys
            1550                1555                1560

Ala Thr Leu Leu Asp Ile Tyr Lys Thr Gly Glu Ala Val Ala
                1565                1570

Glu Lys Asp Ser Glu Ile Thr Phe Ile Lys Lys Val Thr Cys
1575                1580                1585

Thr Asn Ala Glu Leu Val Lys Gly Arg Gln Tyr Leu Ile Met
    1590                1595                1600

Gly Lys Glu Ala Leu Gln Ile Lys Tyr Asn Phe Ser Phe Arg
        1605                1610                1615

Tyr Ile Tyr Pro Leu Asp Ser Leu Thr Trp Ile Glu Tyr Trp
            1620                1625                1630

Pro Arg Asp Thr Thr Cys Ser Ser Cys Gln Ala Phe Leu Ala
                1635                1640

Asn Leu Asp Glu Phe Ala Glu Asp Ile Phe Leu Asn Gly Cys
1645                1650                1655
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 81,
Please delete lines 18-47, and insert therefor:

```
ATG GCC GAC ATC CAG ATG ACT CAG TCT CCA                    30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                  10

GCT TCA CTG TCT GCA TCT GTG GGA GAA ACT                    60
Ala Ser Leu Ser Ala Ser Val Gly Glu Thr
            15                  20

GTC ACC ATC ACA TGT GGA GCA AGT GAG AAT                    90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
            25                  30

ATT TAC GGT GCT TTA AAT TGG TAT CAG CGG                   120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Arg
            35                  40

AAA CAG GGA AAA TCT CCT CAG CTC CTG ATC                   150
Lys Gln Gly Lys Ser Pro Gln Leu Leu Ile
            45                  50

TAT GGT GCA ACC AAC TTG GCA GAT GGC ATG                   180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Met
            55                  60

TCA TCG AGG TTC AGT GGC AGT GGA TCT GGT                   210
Ser Ser Arg Phe Ser Gly Ser Gly Ser Gly
            65                  70

AGA CAG TAT TAT CTC AAG ATC AGT AGC CTG                   240
Arg Gln Tyr Tyr Leu Lys Ile Ser Ser Leu
            75                  80

CAT CCT GAC GAT GTT GCA ACG TAT TAC TGT                   270
His Pro Asp Asp Val Ala Thr Tyr Tyr Cys
            85                  90

CAA AAT GTG TTA AAT ACT CCT CTC ACG TTC                   300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
            95                 100
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 81,
Please delete line 48, through column 83, line 21, and insert therefor:

```
GGT GCT GGG ACC AAG TTG GAG CTG AAA CGG                330
Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg
                105                 110

ACC GGA GGT GGC GGG TCG GGT GGC GGG GGA                360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCG GGT GGC GGA GGG TCG CAG GTT CAG CTG                390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

CAG CAG TCT GGA GCC GAG CTG ATG AAG CCT                420
Gln Gln Ser Gly Ala Glu Leu Met Lys Pro
                135                 140

GGG GCC TCA GTG AAG ATG TCC TGC AAG GCT                450
Gly Ala Ser Val Lys Met Ser Cys Lys Ala
                145                 150

ACT GGC TAC ATA TTC AGT AAC TAC TGG ATA                480
Thr Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAG TGG ATA AAG CAG AGG CCT GGA CAT GGC                510
Gln Trp Ile Lys Gln Arg Pro Gly His Gly
                165                 170

CTT GAG TGG ATT GGT GAG ATT TTA CCT GGA                540
Leu Glu Trp Ile Gly Glu Ile Leu Pro Gly
                175                 180

AGT GGT TCT ACT GAG TAC ACT GAG AAC TTC                570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
                185                 190

AAG GAC AAG GCC GCA TTC ACT GCA GAT ACA                600
Lys Asp Lys Ala Ala Phe Thr Ala Asp Thr
                195                 200
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 83,

Please delete lines 22-36, and insert therefor:

```
TCC TCC AAC ACA GCC TAC ATG CAA CTC AGC                    630
Ser Ser Asn Thr Ala Tyr Met Gln Leu Ser
                205                 210

AGC CTG ACA TCA GAG GAC TCT GCC GTC TAT                    660
Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr
                215                 220

TAC TGT GCA AGA TAT TTC TTC GGT AGT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCC AAC TGG TAC TTC GAT GTC TGG GGC GCA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Ala
                235                 240

GGG ACC ACG GTC ACC GTC TCC TCA TGA                        747
Gly Thr Thr Val Thr Val Ser Ser
                245
```

Please delete lines 46-57, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                     30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                     60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                     90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
                25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CGT                    120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Arg
                35                  40
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 85,

Please delete lines 1-30, and insert therefor:

```
AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                    150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                    180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
                55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                    210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                    240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                    270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                    300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                95                  100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 85,
Please delete lines 31-60, and insert therefor:

```
GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                    570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
                185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
                195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 87,

Please delete lines 1-3, and insert therefor:

```
GGA ACC CTG GTC ACT GTC TCG AGC TGA                              747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

Please delete lines 13-36, and insert therefor:

```
ATG GGA ATC CAA GGA GGG TCT GTC CTG TTC                           30
Met Gly Ile Gln Gly Gly Ser Val Leu Phe
-25                 -20

GGG CTG CTG CTC GTC CTG GCT GTC TTC TGC                           60
Gly Leu Leu Leu Val Leu Ala Val Phe Cys
-15                 -10

CAT TCA GGT CAT AGC CTG CAG GAC ATC CAG                           90
His Ser Gly His Ser Leu Gln Asp Ile Gln
-5                   1               5

ATG ACT CAG TCT CCA GCT TCA CTG TCT GCA                          120
Met Thr Gln Ser Pro Ala Ser Leu Ser Ala
                10                  15

TCT GTG GGA GAA ACT GTC ACC ATC ACA TGT                          150
Ser Val Gly Glu Thr Val Thr Ile Thr Cys
                20                  25

GGA GCA AGT GAG AAT ATT TAC GGT GCT TTA                          180
Gly Ala Ser Glu Asn Ile Tyr Gly Ala Leu
                30                  35

AAT TGG TAT CAG CGG AAA CAG GGA AAA TCT                          210
Asn Trp Tyr Gln Arg Lys Gln Gly Lys Ser
                40                  45

CCT CAG CTC CTG ATC TAT GGT GCA ACC AAC                          240
Pro Gln Leu Leu Ile Tyr Gly Ala Thr Asn
                50                  55
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 87,

Please delete line 37, through column 89, line 9, and insert therefor:

```
TTG GCA GAT GGC ATG TCA TCG AGG TTC AGT                    270
Leu Ala Asp Gly Met Ser Ser Arg Phe Ser
                60                  65

GGC AGT GGA TCT GGT AGA CAG TAT TAT CTC                    300
Gly Ser Gly Ser Gly Arg Gln Tyr Tyr Leu
                70                  75

AAG ATC AGT AGC CTG CAT CCT GAC GAT GTT                    330
Lys Ile Ser Ser Leu His Pro Asp Asp Val
                80                  85

GCA ACG TAT TAC TGT CAA AAT GTG TTA AAT                    360
Ala Thr Tyr Tyr Cys Gln Asn Val Leu Asn
                90                  95

ACT CCT CTC ACG TTC GGT GCT GGG ACC AAG                    390
Thr Pro Leu Thr Phe Gly Ala Gly Thr Lys
            100                 105

TTG GAG CTG AAA CGA ACT GTG GCT GCA CCA                    420
Leu Glu Leu Lys Arg Thr Val Ala Ala Pro
            110                 115

TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG                    450
Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
            120                 125

CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG                    480
Gln Leu Lys Ser Gly Thr Ala Ser Val Val
            130                 135

TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG                    510
Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu
            140                 145

GCC AAA GTA CAG TGG AAG GTG GAT AAC GCC                    540
Ala Lys Val Gln Trp Lys Val Asp Asn Ala
            150                 155
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 89,

Please delete lines 10-29, and insert therefor:

```
CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC                    570
Leu Gln Ser Gly Asn Ser Gln Glu Ser Val
                160                 165

ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC                    600
Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
                170                 175

AGC CTC AGC AGC ACC CTG ACG CTG AGC AAA                    630
Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys
                180                 185

GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC                    660
Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
                190                 195

TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG                    690
Cys Glu Val Thr His Gln Gly Leu Ser Ser
                200                 205

CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG                    720
Pro Val Thr Lys Ser Phe Asn Arg Gly Glu
                210                 215

TGT TAG                                                    726
Cys
```

Please delete lines 39-44, and insert therefor:

```
ATG AAA TGG AGC TGG GTT ATT CTC TTC CTC                     30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
            -15                 -10

CTG TCA GTA ACT GCA GGT GTC CAC TCC CAG                     60
Leu Ser Val Thr Ala Gly Val His Ser Gln
            -5                   1
```

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 89,
Please delete line 45, through column 91, line 15, and insert therefor:

```
GTT CAG CTG CAG CAG TCT GGA GCT GAG CTG                    90
Val Gln Leu Gln Gln Ser Gly Ala Glu Leu
              5                  10

ATG AAG CCT GGG GCC TCA GTG AAG ATG TCC                   120
Met Lys Pro Gly Ala Ser Val Lys Met Ser
             15                  20

TGC AAG GCT ACT GGC TAC ATA TTC AGT AAC                   150
Cys Lys Ala Thr Gly Tyr Ile Phe Ser Asn
             25                  30

TAC TGG ATA CAG TGG ATA AAG CAG AGG CCT                   180
Tyr Trp Ile Gln Trp Ile Lys Gln Arg Pro
             35                  40

GGA CAT GGC CTT GAG TGG ATT GGT GAG ATT                   210
Gly His Gly Leu Glu Trp Ile Gly Glu Ile
             45                  50

TTA CCT GGA AGT GGT TCT ACT GAG TAC ACT                   240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Thr
             55                  60

GAG AAC TTC AAG GAC AAG GCC GCA TTC ACT                   270
Glu Asn Phe Lys Asp Lys Ala Ala Phe Thr
             65                  70

GCA GAT ACA TCC TCC AAC ACA GCC TAC ATG                   300
Ala Asp Thr Ser Ser Asn Thr Ala Tyr Met
             75                  80

CAA CTC AGC AGC CTG ACA TCA GAG GAC TCT                   330
Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser
             85                  90

GCC GTC TAT TAC TGT GCA AGA TAT TTC TTC                   360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
             95                 100
```

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 91,
Please delete lines 16-45, and insert therefor:

```
GGT AGT AGC CCC AAC TGG TAC TTC GAT GTC                    390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
            105                 110

TGG GGC GCA GGG ACC ACG GTC ACC GTC TCC                    420
Trp Gly Ala Gly Thr Thr Val Thr Val Ser
            115                 120

TCA GCC TCC ACC AAG GGC CCA TCG GTC TTC                    450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
            125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT                    480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
            135                 140

GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC                    510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
            145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG                    540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
            155                 160

TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC                    570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
            165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC                    600
Val His Thr Phe Pro Ala Val Leu Gln Ser
            175                 180

TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG                    630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
            185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG                    660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
            195                 200
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 91,

Please delete lines 46-54, and insert therefor:

```
ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC                690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
            205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG                720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
            215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA                750
Pro Lys Ser Cys Asp Lys Thr His Thr
            225
```

Column 93

Please delete lines 4-21, and insert therefor:

```
ATG AAG TGG AGC TGG GTT ATT CTC TTC CTC                 30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
                -15                 -10

CTG TCA GTA ACT GCC GGC GTC CAC TCC CAA                 60
Leu Ser Val Thr Ala Gly Val His Ser Gln
                 -5                  1

GTC CAA CTG GTG CAA TCC GGC GCC GAG GTC                 90
Val Gln Leu Val Gln Ser Gly Ala Glu Val
              5                  10

AAG AAG CCA GGG GCC TCA GTC AAA GTG TCC                120
Lys Lys Pro Gly Ala Ser Val Lys Val Ser
             15                  20

TGT AAA GCT AGC GGC TAT ATT TTT TCT AAT                150
Cys Lys Ala Ser Gly Tyr Ile Phe Ser Asn
             25                  30

TAT TGG ATT CAA TGG GTG CGT CAG GCC CCC                180
Tyr Trp Ile Gln Trp Val Arg Gln Ala Pro
             35                  40
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 93,
Please delete lines 22-51, and insert therefor:

```
GGG CAG GGC CTG GAA TGG ATG GGT GAG ATC                    210
Gly Gln Gly Leu Glu Trp Met Gly Glu Ile
            45                  50

TTA CCG GGC TCT GGT AGC ACC GAA TAT GCC                    240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Ala
            55                  60

CAA AAA TTC CAG GGC CGT GTT ACT ATG ACT                    270
Gln Lys Phe Gln Gly Arg Val Thr Met Thr
            65                  70

GCG GAC ACT TCG ACT AGT ACA GCC TAC ATG                    300
Ala Asp Thr Ser Thr Ser Thr Ala Tyr Met
            75                  80

GAG CTC TCC AGC CTG CGA TCG GAG GAC ACG                    330
Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr
            85                  90

GCC GTC TAT TAT TGC GCG CGT TAT TTT TTT                    360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
            95                  100

GGT TCT AGC CCG AAT TGG TAT TTT GAT GTT                    390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
            105                 110

TGG GGT CAA GGA ACC CTG GTC ACT GTC TCG                    420
Trp Gly Gln Gly Thr Leu Val Thr Val Ser
            115                 120

AGC GCC TCC ACC AAG GGC CCA TCG GTC TTC                    450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
            125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT                    480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
            135                 140
```

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 93,
Please delete line 52, through column 95, line 27, and insert therefor:

```
GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC                    510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
            145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG                    540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
            155                 160

TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC                    570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
            165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC                    600
Val His Thr Phe Pro Ala Val Leu Gln Ser
            175                 180

TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG                    630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
            185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG                    660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
            195                 200

ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC                    690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
            205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG                    720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
            215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA                    750
Pro Lys Ser Cys Asp Lys Thr His Thr
            225                 230
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 95,

Please delete line 31, through column 97, line 3, and insert therefor:

```
ATG AAG TGG AGC TGG GTT ATT CTC TTC CTC                    30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
            -15                 -10

CTG TCA GTA ACT GCC GGC GTC CAC TCC CAA                    60
Leu Ser Val Thr Ala Gly Val His Ser Gln
            -5                   1

GTC CAA CTG GTG CAA TCC GGC GCC GAG GTC                    90
Val Gln Leu Val Gln Ser Gly Ala Glu Val
            5                   10

AAG AAG CCA GGG GCC TCA GTC AAA GTG TCC                   120
Lys Lys Pro Gly Ala Ser Val Lys Val Ser
            15                  20

TGT AAA GCT AGC GGC TAT ATT TTT TCT AAT                   150
Cys Lys Ala Ser Gly Tyr Ile Phe Ser Asn
            25                  30

TAT TGG ATT CAA TGG GTG CGT CAG GCC CCC                   180
Tyr Trp Ile Gln Trp Val Arg Gln Ala Pro
            35                  40

GGG CAG GGC CTG GAA TGG ATG GGT GAG ATC                   210
Gly Gln Gly Leu Glu Trp Met Gly Glu Ile
            45                  50

TTA CCG GGC TCT GGT AGC ACC GAA TAT ACC                   240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Thr
            55                  60

GAA AAT TTT AAA GAC CGT GTT ACT ATG ACG                   270
Glu Asn Phe Lys Asp Arg Val Thr Met Thr
            65                  70

CGT GAC ACT TCG ACT AGT ACA GTA TAC ATG                   300
Arg Asp Thr Ser Thr Ser Thr Val Tyr Met
            75                  80
```

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 97,

Please delete lines 4-33, and insert therefor:

```
GAG CTC TCC AGC CTG CGA TCG GAG GAC ACG                    330
Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr
             85                  90

GCC GTC TAT TAT TGC GCG CGT TAT TTT TTT                    360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
             95                 100

GGT TCT AGC CCG AAT TGG TAT TTT GAT GTT                    390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
            105                 110

TGG GGT CAA GGA ACC CTG GTC ACT GTC TCG                    420
Trp Gly Gln Gly Thr Leu Val Thr Val Ser
            115                 120

AGC GCC TCC ACC AAG GGC CCA TCG GTC TTC                    450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
            125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT                    480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
            135                 140

GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC                    510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
            145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG                    540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
            155                 160

TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC                    570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
            165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC                    600
Val His Thr Phe Pro Ala Val Leu Gln Ser
            175                 180
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 97,
Please delete lines 34-48, and insert therefor:

```
TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG                    630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
            185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG                    660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
            195                 200

ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC                    690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
            205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG                    720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
            215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA                    750
Pro Lys Ser Cys Asp Lys Thr His Thr
            225                 230
```

Column 99,
Please delete lines 2-13, and insert therefor:

```
ATG GGA ATC CAA GGA GGG TCT GTC CTG TTC                     30
Met Gly Ile Gln Gly Gly Ser Val Leu Phe
-25                 -20

GGG CTG CTG CTC GTC CTG GCT GTC TTC TGC                     60
Gly Leu Leu Leu Val Leu Ala Val Phe Cys
-15                 -10

CAT TCA GGT CAT AGC CTG CAG GAT ATC CAG                     90
His Ser Gly His Ser Leu Gln Asp Ile Gln
-5                   1                   5

ATG ACC CAG TCC CCG TCC TCC CTG TCC GCC                    120
Met Thr Gln Ser Pro Ser Ser Leu Ser Ala
                10                  15
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 99,

Please delete lines 14-43, and insert therefor:

```
TCT GTG GGC GAT AGG GTC ACC ATC ACC TGC                    150
Ser Val Gly Asp Arg Val Thr Ile Thr Cys
                  20                  25

GGC GCC AGC GAA AAC ATC TAT GGC GCG CTG                    180
Gly Ala Ser Glu Asn Ile Tyr Gly Ala Leu
                  30                  35

AAC TGG TAT CAA CGT AAA CCT GGG AAA GCT                    210
Asn Trp Tyr Gln Arg Lys Pro Gly Lys Ala
                  40                  45

CCG AAG CTT CTG ATT TAC GGT GCG ACG AAC                    240
Pro Lys Leu Leu Ile Tyr Gly Ala Thr Asn
                  50                  55

CTG GCA GAT GGA GTC CCT TCT CGC TTC TCT                    270
Leu Ala Asp Gly Val Pro Ser Arg Phe Ser
                  60                  65

GGA TCC GGC TCC GGA ACG GAT TAC ACT CTG                    300
Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu
                  70                  75

ACC ATC AGC AGT CTG CAA CCT GAG GAC TTC                    330
Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe
                  80                  85

GCT ACG TAT TAC TGT CAG AAC GTT TTA AAT                    360
Ala Thr Tyr Tyr Cys Gln Asn Val Leu Asn
                  90                  95

ACT CCG TTG ACT TTC GGA CAG GGT ACC AAG                    390
Thr Pro Leu Thr Phe Gly Gln Gly Thr Lys
                 100                 105

GTG GAA ATA AAA CGA ACT GTG GCT GCA CCA                    420
Val Glu Ile Lys Arg Thr Val Ala Ala Pro
                 110                 115
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 99,
Please delete line 44, through column 101, line 17, and insert therefor:

```
TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG                    450
Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
                120                 125

CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG                    480
Gln Leu Lys Ser Gly Thr Ala Ser Val Val
                130                 135

TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG                    510
Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu
                140                 145

GCC AAA GTA CAG TGG AAG GTG GAT AAC GCC                    540
Ala Lys Val Gln Trp Lys Val Asp Asn Ala
                150                 155

CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC                    570
Leu Gln Ser Gly Asn Ser Gln Glu Ser Val
                160                 165

ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC                    600
Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
                170                 175

AGC CTC AGC AGC ACC CTG ACG CTG AGC AAA                    630
Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys
                180                 185

GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC                    660
Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
                190                 195

TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG                    690
Cys Glu Val Thr His Gln Gly Leu Ser Ser
                200                 205

CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG                    720
Pro Val Thr Lys Ser Phe Asn Arg Gly Glu
                210                 215

TGT TAG                                                    726
Cys
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 101,

Please delete lines 28-57, and insert therefor:

```
ATG GGA ATC CAA GGA GGG TCT GTC CTG TTC                    30
Met Gly Ile Gln Gly Gly Ser Val Leu Phe
-25                 -20

GGG CTG CTG CTC GTC CTG GCT GTC TTC TGC                    60
Gly Leu Leu Leu Val Leu Ala Val Phe Cys
-15                 -10

CAT TCA GGT CAT AGC CTG CAG GAT ATC CAG                    90
His Ser Gly His Ser Leu Gln Asp Ile Gln
-5                   1                 5

ATG ACC CAG TCC CCG TCC TCC CTG TCC GCC                   120
Met Thr Gln Ser Pro Ser Ser Leu Ser Ala
                10                  15

TCT GTG GGC GAT AGG GTC ACC ATC ACC TGC                   150
Ser Val Gly Asp Arg Val Thr Ile Thr Cys
                20                  25

GGC GCC AGC GAA AAC ATC TAT GGC GCG CTG                   180
Gly Ala Ser Glu Asn Ile Tyr Gly Ala Leu
                30                  35

AAC TGG TAT CAA CGT AAA CCT GGG AAA GCT                   210
Asn Trp Tyr Gln Arg Lys Pro Gly Lys Ala
                40                  45

CCG AAG CTT CTG ATT TAC GGT GCG ACG AAC                   240
Pro Lys Leu Leu Ile Tyr Gly Ala Thr Asn
                50                  55

CTG GCA GAT GGA GTC CCT TCT CGC TTC TCT                   270
Leu Ala Asp Gly Val Pro Ser Arg Phe Ser
                60                  65

GGA TCC GGC TCC GGA ACG GAT TTC ACT CTG                   300
Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu
                70                  75
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 103,

Please delete lines 1-30, and insert therefor:

```
ACC ATC AGC AGT CTG CAG CCT GAA GAC TTC                    330
Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe
                 80                  85

GCT ACG TAT TAC TGT CAG AAC GTT TTA AAT                    360
Ala Thr Tyr Tyr Cys Gln Asn Val Leu Asn
                 90                  95

ACT CCG TTG ACT TTC GGA CAG GGT ACC AAG                    390
Thr Pro Leu Thr Phe Gly Gln Gly Thr Lys
                100                 105

GTG GAA ATA AAA CGA ACT GTG GCT GCA CCA                    420
Val Glu Ile Lys Arg Thr Val Ala Ala Pro
                110                 115

TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG                    450
Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
                120                 125

CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG                    480
Gln Leu Lys Ser Gly Thr Ala Ser Val Val
                130                 135

TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG                    510
Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu
                140                 145

GCC AAA GTA CAG TGG AAG GTG GAT AAC GCC                    540
Ala Lys Val Gln Trp Lys Val Asp Asn Ala
                150                 155

CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC                    570
Leu Gln Ser Gly Asn Ser Gln Glu Ser Val
                160                 165

ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC                    600
Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
                170                 175
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 103,
Please delete lines 31-44, and insert therefor:

```
AGC CTC AGC AGC ACC CTG ACG CTG AGC AAA                    630
Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys
                180                 185

GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC                    660
Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
                190                 195

TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG                    690
Cys Glu Val Thr His Gln Gly Leu Ser Ser
                200                 205

CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG                    720
Pro Val Thr Lys Ser Phe Asn Arg Gly Glu
                210                 215

TGT TAG                                                    726
Cys
```

Please delete line 55, through column 105, line 9, and insert therefor:

```
ATG GAC ATG AGG GTC CCC GCT CAG CTC CTG                     30
Met Asp Met Arg Val Pro Ala Gln Leu Leu
            -20                 -15

GGG CTC CTG CTA CTC TGG CTC CGA GGT GCC                     60
Gly Leu Leu Leu Leu Trp Leu Arg Gly Ala
            -10                 -5

AGA TGT GAT ATC CAG ATG ACC CAG TCC CCG                     90
Arg Cys Asp Ile Gln Met Thr Gln Ser Pro
          1                 5

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                    120
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
     10                  15
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 105,

Please delete lines 10-39, and insert therefor:

```
GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                    150
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
     20                  25

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                    180
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
     30                  35

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                    210
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
     40                  45

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                    240
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
     50                  55

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                    270
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
     60                  65

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                    300
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
     70                  75

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                    330
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
     80                  85

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                    360
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
     90                  95

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGA                    390
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
    100                 105

ACT GTG GCT GCA CCA TCT GTC TTC ATC TTC                    420
Thr Val Ala Ala Pro Ser Val Phe Ile Phe
    110                 115
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 105,
Please delete line 40, through column 107, line 9, and insert therefor:

```
CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA                    450
Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
    120                 125

ACT GCC TCT GTT GTG TGC CTG CTG AAT AAC                    480
Thr Ala Ser Val Val Cys Leu Leu Asn Asn
    130                 135

TTC TAT CCC AGA GAG GCC AAA GTA CAG TGG                    510
Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp
    140                 145

AAG GTG GAT AAC GCC CTC CAA TCG GGT AAC                    540
Lys Val Asp Asn Ala Leu Gln Ser Gly Asn
    150                 155

TCC CAG GAG AGT GTC ACA GAG CAG GAC AGC                    570
Ser Gln Glu Ser Val Thr Glu Gln Asp Ser
    160                 165

AAG GAC AGC ACC TAC AGC CTC AGC AGC ACC                    600
Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr
    170                 175

CTG ACG CTG AGC AAA GCA GAC TAC GAG AAA                    630
Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys
    180                 185

CAC AAA GTC TAC GCC TGC GAA GTC ACC CAT                    660
His Lys Val Tyr Ala Cys Glu Val Thr His
    190                 195

CAG GGC CTG AGC TCG CCC GTC ACA AAG AGC                    690
Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
    200                 205

TTC AAC AGG GGA GAG TGT TAG                               711
Phe Asn Arg Gly Glu Cys
    210
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 107,
Please delete lines 20-49, and insert therefor:

```
ATG AAG TGG AGC TGG GTT ATT CTC TTC CTC                    30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
               -15                 -10

CTG TCA GTA ACT GCC GGC GTC CAC TCC CAA                    60
Leu Ser Val Thr Ala Gly Val His Ser Gln
                -5                   1

GTC CAA CTG GTG CAA TCC GGC GCC GAG GTC                    90
Val Gln Leu Val Gln Ser Gly Ala Glu Val
             5                   10

AAG AAG CCA GGG GCC TCA GTC AAA GTG TCC                   120
Lys Lys Pro Gly Ala Ser Val Lys Val Ser
            15                  20

TGT AAA GCT AGC GGC TAT ATT TTT TCT AAT                   150
Cys Lys Ala Ser Gly Tyr Ile Phe Ser Asn
            25                  30

TAT TGG ATT CAA TGG GTG CGT CAG GCC CCC                   180
Tyr Trp Ile Gln Trp Val Arg Gln Ala Pro
            35                  40

GGG CAG GGC CTG GAA TGG ATG GGT GAG ATC                   210
Gly Gln Gly Leu Glu Trp Met Gly Glu Ile
            45                  50

TTA CCG GGC TCT GGT AGC ACC GAA TAT GCC                   240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Ala
            55                  60

CAA AAA TTC CAG GGC CGT GTT ACT ATG ACT                   270
Gln Lys Phe Gln Gly Arg Val Thr Met Thr
            65                  70

CGT GAC ACT TCG ACT AGT ACA GTA TAC ATG                   300
Arg Asp Thr Ser Thr Ser Thr Val Tyr Met
            75                  80
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 107,
Please delete line 50, through column 109, line 21, and insert therefor:

```
GAG CTC TCC AGC CTG CGA TCG GAG GAC ACG                    330
Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr
             85                  90

GCC GTC TAT TAT TGC GCG CGT TAT TTT TTT                    360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
             95                 100

GGT TCT AGC CCG AAT TGG TAT TTT GAT GTT                    390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
            105                 110

TGG GGT CAA GGA ACC CTG GTC ACT GTC TCG                    420
Trp Gly Gln Gly Thr Leu Val Thr Val Ser
            115                 120

AGC GCC TCC ACC AAG GGC CCA TCG GTC TTC                    450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
            125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT                    480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
            135                 140

GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC                    510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
            145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG                    540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
            155                 160

TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC                    570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
            165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC                    600
Val His Thr Phe Pro Ala Val Leu Gln Ser
            175                 180
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 109,

Please delete lines 22-36, and insert therefor:

```
TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG                    630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
            185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG                    660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
            195                 200

ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC                    690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
            205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG                    720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
            215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA                    750
Pro Lys Ser Cys Asp Lys Thr His Thr
            225                 230
```

Please delete lines 47-58, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                     30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                     60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
            15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                     90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
            25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                    120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
            35                  40
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 111,
Please delete lines 1-30, and insert therefor:

```
AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                    150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                 45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                    180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
                 55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                    210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                 65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                    240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                 75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                    270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                 85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                    300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                 95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 111,
Please delete lines 31-60, and insert therefor:

```
GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT GCC CAA AAA TTC                    570
Ser Gly Ser Thr Glu Tyr Ala Gln Lys Phe
                185                 190

CAG GGC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Gln Gly Arg Val Thr Met Thr Arg Asp Thr
                195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 113,
Please delete lines 1-3, and insert therefor:

```
GGA ACC CTG GTC ACT GTC TCG AGC TGA                              747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

Column 119,
Please delete lines 2-25, and insert therefor:

```
ATG GCC AAT ATT GTG CTG ACC CAA TCT CCA                           30
Met Ala Asn Ile Val Leu Thr Gln Ser Pro
  1               5                  10

GCT TCT TTG GCT GTG TCT CTA GGG CAG AGG                           60
Ala Ser Leu Ala Val Ser Leu Gly Gln Arg
                15                  20

GCC ACC ATA TCC TGC AGA GCC AGT GAA AGT                           90
Ala Thr Ile Ser Cys Arg Ala Ser Glu Ser
                25                  30

GTT GAT AGT TAT GAC AAT AGT TTT ATG CAC                          120
Val Asp Ser Tyr Asp Asn Ser Phe Met His
                35                  40

TGG TAC CAG CAG AAA CCA GGA CAG CCA CCC                          150
Trp Tyr Gln Gln Lys Pro Gly Gln Pro Pro
                45                  50

AAA CTC CTC ATC TTT CTT GCA TCC AAC CTA                          180
Lys Leu Leu Ile Phe Leu Ala Ser Asn Leu
                55                  60

GAA TCT GGG GTC CCT GCC AGG TTC AGT GGC                          210
Glu Ser Gly Val Pro Ala Arg Phe Ser Gly
                65                  70

AGT GGG TCT AGG ACA GAC TTC ACC CTC ACC                          240
Ser Gly Ser Arg Thr Asp Phe Thr Leu Thr
                75                  80
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 119,
Please delete lines 26-55, and insert therefor:

```
ATT GAT CCT GTG GAG GCT GAT GAT GCT GCA                    270
Ile Asp Pro Val Glu Ala Asp Asp Ala Ala
                 85                  90

ACC TAT TAC TGT CAG CAA AAT AAT GAG GTT                   300
Thr Tyr Tyr Cys Gln Gln Asn Asn Glu Val
                 95                 100

CCG AAC ACG TTC GGA GGG GGG ACC AAG CTG                   330
Pro Asn Thr Phe Gly Gly Gly Thr Lys Leu
                105                 110

GAA ATA AAA CGG ACC GGA GGT GGC GGG TCG                   360
Glu Ile Lys Arg Thr Gly Gly Gly Gly Ser
                115                 120

GGT GGC GGG GGA TCG GGT GGC GGA GGG TCG                   390
Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
                125                 130

GAC GTC AAG CTC GTG GAG TCT GGG GGA GAC                   420
Asp Val Lys Leu Val Glu Ser Gly Gly Asp
                135                 140

TTA GTG AAG CTT GGA GGG TCC CTG AAA CTC                   450
Leu Val Lys Leu Gly Gly Ser Leu Lys Leu
                145                 150

TCC TGT GCA GCC TCT GGA TTC ACC TTC AGT                   480
Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
                155                 160

AGC TAT TAT ATG TCT TGG GTT CGC CAG ATT                   510
Ser Tyr Tyr Met Ser Trp Val Arg Gln Ile
                165                 170

TCA GAG AAG AGG CTG GAG TTG GTC GCA GCC                   540
Ser Glu Lys Arg Leu Glu Leu Val Ala Ala
                175                 180
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 119,

Please delete lines 56, through column 121, line 22, and insert therefor:

```
ATT AAT AGT AAT GGT GAT AGC ACC TAC TAT                    570
Ile Asn Ser Asn Gly Asp Ser Thr Tyr Tyr
                185                 190

CCA GAC ACT GTG AAG GGC CGA TTC ACC ATC                    600
Pro Asp Thr Val Lys Gly Arg Phe Thr Ile
                195                 200

TCC AGA GAC AAT GCC AAG AGC ACC CTG GAT                    630
Ser Arg Asp Asn Ala Lys Ser Thr Leu Asp
                205                 210

CTG CAA ATG AGC AGT CTG AAG TCT GAG GAC                    660
Leu Gln Met Ser Ser Leu Lys Ser Glu Asp
                215                 220

ACA GCC TTG TAT TTC TGT GTA AGA GAG ACT                    690
Thr Ala Leu Tyr Phe Cys Val Arg Glu Thr
                225                 230

TAT TAC TAC GGG ATT AGT CCC GTC TTC GAT                    720
Tyr Tyr Tyr Gly Ile Ser Pro Val Phe Asp
                235                 240

GTC TGG GGC ACA GGG ACC ACG GTC ACC GTC                    750
Val Trp Gly Thr Gly Thr Thr Val Thr Val
                245                 250

TCC TCA CTC GAG CAC CAC CAC CAC CAC CAC                    780
Ser Ser Leu Glu His His His His His His
                255                 260

TGA                                                        783
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 121,
Please delete lines 32, through column 123, line 3, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                    30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                    60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                    90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
                25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                   120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
                35                  40

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                   150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                   180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
                55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                   210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                   240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                   270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                   300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                95                 100
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 123,
Please delete lines 4-33, and insert therefor:

```
GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                    570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
                185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
                195                 200
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 123,
Please delete lines 34-48, and insert therefor:

```
TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                        747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

Column 125,
Please delete lines 2-13, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                     30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                     60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20

GTC ACC ATC ACC TGC CGT GCT AGC GAA AAC                     90
Val Thr Ile Thr Cys Arg Ala Ser Glu Asn
                25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                    120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
                35                  40
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 125,
Please delete lines 14-43, and insert therefor:

```
AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                    150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                    180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
                55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                    210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                    240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                    270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                    300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
               105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
               115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
               125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
               135                 140
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 125,
Please delete line 44, through column 127, line 15, and insert therefor:

```
GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT GCC CAA AAA TTC                    570
Ser Gly Ser Thr Glu Tyr Ala Gln Lys Phe
                185                 190

CAG GGC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Gln Gly Arg Val Thr Met Thr Arg Asp Thr
                195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 127,

Please delete lines 16-18, and insert therefor:

```
GGA ACC CTG GTC ACT GTC TCG AGC TGA                              747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

Please delete lines 29-52, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                           30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                           60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                           90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
                25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                          120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
                35                  40

AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                          150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                45                  50

TAC GGT GCG ACG AGC CTG CAG TCT GGA GTC                          180
Tyr Gly Ala Thr Ser Leu Gln Ser Gly Val
                55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                          210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                          240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                75                  80
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 127,
Please delete line 53, through column 129, line 24, and insert therefor:

```
CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                    270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                 85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                    300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                 95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 129,

Please delete lines 25-45, and insert therefor:

```
TCT GGT AGC ACC GAA TAT GCC CAA AAA TTC                  570
Ser Gly Ser Thr Glu Tyr Ala Gln Lys Phe
                185                 190

CAG GGC CGT GTT ACT ATG ACG CGT GAC ACT                  600
Gln Gly Arg Val Thr Met Thr Arg Asp Thr
                195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                  630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                  660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                  690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                  720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                      747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

Please delete line 56, through column 131, line 3, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                   30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                   60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 131,
Please delete lines 4-33, and insert therefor:

```
GTC ACC ATC ACC TGC CGT GCT AGC GAA AAC                    90
Val Thr Ile Thr Cys Arg Ala Ser Glu Asn
                 25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                   120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
                 35                  40

AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                   150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                 45                  50

TAC GGT GCG ACG AGC CTG CAG TCT GGA GTC                   180
Tyr Gly Ala Thr Ser Leu Gln Ser Gly Val
                 55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                   210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                 65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                   240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                 75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                   270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                 85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                   300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                 95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                   330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                   360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 131,
Please delete line 34, through column 133, line 3, and insert therefor:

```
TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT GCC CAA AAA TTC                570
Ser Gly Ser Thr Glu Tyr Ala Gln Lys Phe
                185                 190

CAG GGC CGT GTT ACT ATG ACG CGT GAC ACT                600
Gln Gly Arg Val Thr Met Thr Arg Asp Thr
                195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 133,

Please delete lines 4-12, and insert therefor:

```
TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                    747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

Please delete lines 22-45, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                 30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                 60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20

GTC ACC ATC ACC TGC CGT GCT AGC GAA AAC                 90
Val Thr Ile Thr Cys Arg Ala Ser Glu Asn
                25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
                35                  40

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
                55                  60
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CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 133,

Please delete line 46, through column 135, line 12, and insert therefor:

```
CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                    210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                 65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                    240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                 75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                    270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                 85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                    300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                 95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 135,
Please delete lines 13-39, and insert therefor:

```
CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                    570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
                185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
                195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                        747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 135,
Please delete line 49, through column 137, line 21, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                    30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                    60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                 15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                    90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
                 25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                   120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
                 35                  40

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                   150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                 45                  50

TAC GGT GCG ACG AGC CTG CAG TCT GGA GTC                   180
Tyr Gly Ala Thr Ser Leu Gln Ser Gly Val
                 55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                   210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                 65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                   240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                 75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                   270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                 85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                   300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                 95                 100
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 137,
Please delete lines 22-51, and insert therefor:

```
GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                    570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
                185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
                195                 200
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 137,
Please delete line 52, through column 139, line 6, and insert therefor:

```
TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                        747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

Column 139,
Please delete lines 16-27, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                     30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                     60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20

GTC ACC ATC ACC TGC CGT GCT AGC GAA AAC                     90
Val Thr Ile Thr Cys Arg Ala Ser Glu Asn
                25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                    120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
                35                  40
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 139,
Please delete lines 28-57, and insert therefor:

```
AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                    150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                 45                  50

TAC GGT GCG ACG AGC CTG CAG TCT GGA GTC                    180
Tyr Gly Ala Thr Ser Leu Gln Ser Gly Val
                 55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                    210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                 65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                    240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                 75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                    270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                 85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                    300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                 95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 141,
Please delete lines 1-30, and insert therefor:

```
GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                    570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
                185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
                195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240
```

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 141,
Please delete lines 31-33, and insert therefor:

```
GGA ACC CTG GTC ACT GTC TCG AGC TGA                    747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

Signed and Sealed this

Nineteenth Day of November, 2002

Attest:

Attesting Officer

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

Sustained response and long-term safety of eculizumab in paroxysmal nocturnal hemoglobinuria

Anita Hill, Peter Hillmen, Stephen J. Richards, Dupe Elebute, Judith C. Marsh, Jason Chan, Christopher F. Mojcik, and Russell P. Rother

Paroxysmal nocturnal hemoglobinuria (PNH) is a hematologic disorder characterized by clonal expansion of red blood cells (RBCs) lacking the ability to inhibit complement-mediated hemolysis. Eculizumab, a humanized monoclonal antibody that binds the C5 complement protein, blocks serum hemolytic activity. This study evaluated the long-term safety and efficacy of eculizumab in 11 patients with PNH during an open-label extension trial. After completion of an initial 12-week study, all patients chose to participate in the 52-week extension study. Eculizumab, administered at 900 mg every 12 to 14 days, was sufficient to completely and consistently block complement activity in all patients. A dramatic reduction in hemolysis was maintained throughout the study, with a decrease in lactate dehydrogenase (LDH) levels from 3110.7 IU/L before treatment to 622.4 IU/L ($P = .002$). The proportion of PNH type III RBCs increased from 36.7% at baseline to 58.4% ($P = .005$). The paroxysm rate of days with gross evidence of hemoglobinuria per patient each month decreased from 3.0 during screening to 0.2 ($P < .001$) during treatment. The median transfusion rate decreased from 1.8 U per patient each month before eculizumab treatment to 0.3 U per patient each month ($P = .001$) during treatment. Statistically significant improvements in quality-of-life measures were also maintained during the extension study. Eculizumab continued to be safe and well tolerated, and all patients completed the study. The close relationship between sustained terminal complement inhibition, hemolysis, and symptoms was demonstrated. (Blood. 2005; 106:2559-2565)

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a hematopoietic stem-cell disorder characterized by red blood cell (RBC) destruction, anemia, hemoglobinuria, and thrombosis. The intravascular hemolysis in PNH is continuous, with episodes of dark urine, or paroxysms, occurring at times of particularly brisk hemolysis. Ongoing hemolysis and/or insufficient hematopoiesis often result in transfusion dependence. Hemolysis in patients with PNH can be monitored by levels of the enzyme lactate dehydrogenase (LDH), which is typically elevated and can exceed 20 times the upper limit of normal during severe paroxysms.[1-3] There is no effective treatment for the ongoing hemolysis in PNH.

PNH results from the clonal expansion of somatically mutated hematopoietic stem cells. The predominant mutation results in a functional deficiency in phosphatidylinositol glycan class A (PIG-A), a protein that is critical for the biosynthesis of the glycosylphosphatidylinositol (GPI) anchor, a mechanism by which various proteins are attached to the cell membrane.[4,5] Consequently, there is a partial (type II) or complete (type III) deficiency of GPI-anchored proteins on the surfaces of PNH hematopoietic stem cells and their progeny. Two such proteins are the complement inhibitors CD55 and CD59. CD55 inhibits complement at the level of C3, whereas CD59 prevents terminal complement components from forming the hemolytic membrane pore (C5b-9).[6-8] Deficiency of these complement inhibitors renders PNH RBCs sensitive to complement-mediated lysis.[7]

Eculizumab is a humanized monoclonal antibody that specifically targets the complement protein C5 and prevents its cleavage.[9] C5 is the point at which the pathways of complement activation converge, and it is the first protein of terminal complement assembly. Complement inhibition at this stage blocks the generation of C5a and the formation of C5b-9 while it preserves early complement components that are critical for the clearance of microorganisms and immune complexes.[10]

We previously reported the outcome of an open-label study of eculizumab in patients with PNH.[2] Results of this 12-week study demonstrated a dramatic reduction in hemolysis and a concomitant increase in the proportion of PNH type III RBCs. In addition, this initial study showed a marked decrease in the rates of paroxysms and blood transfusions and an improvement in quality of life. Here we report the results of a 1-year follow-up study designed to assess the long-term efficacy and safety of eculizumab in patients with PNH.

From the Department of Haematology, Leeds Teaching Hospitals NHS Trust, Leeds, United Kingdom; the Department of Haematology, Saint George's Hospital Medical School, London, United Kingdom; and Alexion Pharmaceuticals, Inc, Cheshire, CT.

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Supported by Alexion Pharmaceuticals, Inc.

Several of the authors (J.C., C.F.M., R.P.R.) are employed by Alexion, whose potential product was studied in the present work. P.H. reports serving as a consultant to Alexion, and A.H. and P.H. have each received an educational grant from the company.

An Inside *Blood* analysis of this article appears at the front of this issue.

Reprints: Anita Hill, Department of Haematology, Leeds Teaching Hospitals NHS Trust, Great George St, Leeds, LS1 3EX, United Kingdom; e-mail: anitahill@nhs.net.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

Exhibit F

Patients, materials, and methods

Trial design

The acute-phase study was an initial 12-week, open-label trial of eculizumab in 11 patients with PNH and has been described previously in detail.[2] The current study was an open-label extension of that acute-phase study. All 11 patients from the acute-phase study enrolled in the extension study. Patients were allowed concomitant therapy—with the exception of whole blood, which contains C5—at the discretion of their treating physicians. Two of 11 patients had a history of thrombosis before eculizumab treatment, and 6 of 11 patients were on warfarin therapy before and during the trial.

The trial was approved by the Leeds (West) Research Ethics Committee, United Kingdom, and was performed according to the International Conference on Harmonisation and Good Clinical Practice Standards. All patients gave written informed consent and were treated with eculizumab.

Eculizumab administration

All patients entered the extension study on a maintenance dose of eculizumab (at the conclusion of the acute-phase study). This maintenance dose of 900 mg intravenously every 14 days was continued throughout the extension study period. Two patients, however, required that the dosing interval be shortened to every 12 days so that consistent and complete complement inhibition could be maintained.

Clinical investigations

As in the acute-phase study, data were obtained in the open-label extension study on the pharmacokinetics (PK), pharmacodynamics (PD), and immunogenicity of eculizumab, indicators of hemolysis, PNH clone size, paroxysm and transfusion rates, and quality-of-life measurements. The trigger for transfusion before and during the study remained unchanged for each patient and was based on a combination of hemoglobin levels and the occurrence of symptoms resulting from anemia, hemolysis, or both. This information has been described in detail elsewhere.[2]

Statistical analysis

Biochemical values were compared using the paired Student *t* test, change of transfusion and paroxysm rates were analyzed using the Wilcoxon signed rank test, quality-of-life measurements were analyzed using mixed-effect analysis of variance, and comparison of the number of days with paroxysms was analyzed using the Fisher exact test.

Results

Pharmacokinetics and pharmacodynamics

In the acute-phase study, 10 of 11 patients maintained sufficient levels of eculizumab (≥ 35 μg/mL) for terminal complement to be sufficiently inhibited (≤ 20% serum hemolytic activity) for the duration of the 12-week treatment period (Figure 1, weeks 0-12). During the extension study, 9 of 11 patients continued to show complete complement blockade throughout the 52-week treatment period (Figure 1, weeks 12-64). The 2 patients whose serum eculizumab levels decreased below 35 μg/mL (Figure 1A, lines 1-2) showed a return of serum hemolytic activity (Figure 1B, lines

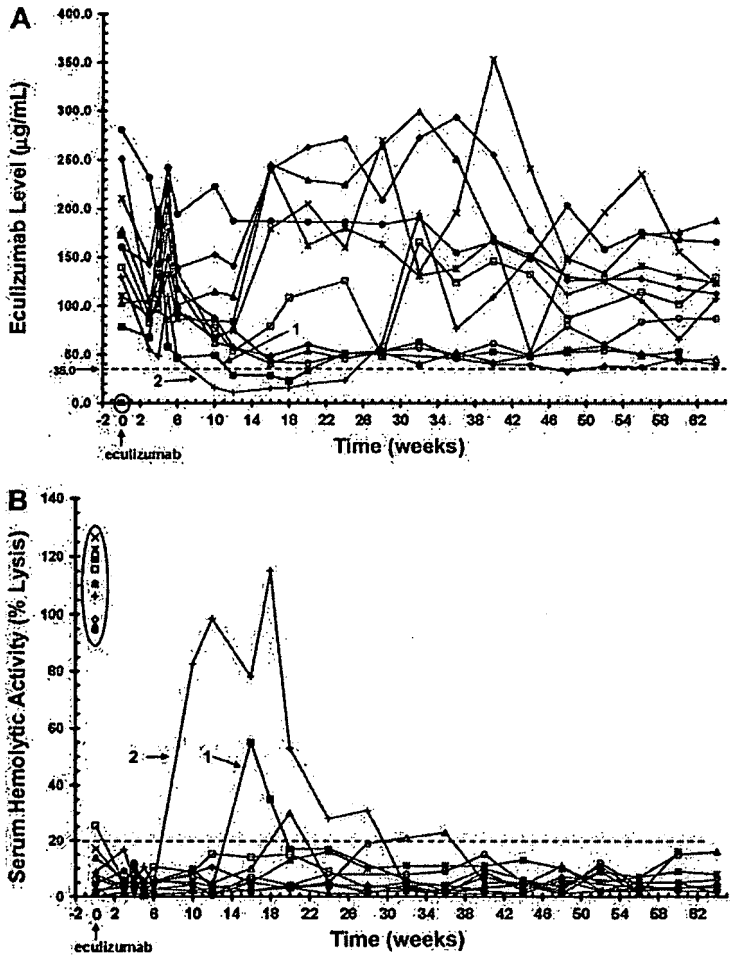


Figure 1. PK and PD analyses of eculizumab in patients with PNH. Initiation of eculizumab dosing is indicated at time 0 on the x axis. (A) Serum levels of eculizumab in 11 patients with PNH during the 64 weeks of treatment. The dashed line indicates the level of eculizumab required to completely block complement activity (≥ 35 μg/mL). Time 0 shows levels of eculizumab before (encircled) and 1 hour after dosing, whereas all other time points represent trough values. Two patients with trough levels of eculizumab below 35 μg/mL during the maintenance dosing are identified (patients 1 and 2). (B) Serum hemolytic activity (PD) during the 64-week treatment period, as determined by the ability of serum to lyse antibody-presensitized chicken erythrocytes. The dashed line indicates the percentage of hemolytic activity at which complement is considered effectively inhibited (≤ 20%). Time 0 shows serum hemolytic activity before (encircled) and 1 hour after dosing, whereas all other time points represent trough values. Two patients with trough serum hemolytic activity values above 20% are identified (patients 1 and 2).

1-2). However, a reduction in the dosing interval from 900 mg every 14 days to 900 mg every 12 days (initiated between weeks 18 and 24) in the 2 breakthrough patients was adequate to keep trough levels of eculizumab higher than 35 μg/mL (Figure 1A), thereby completely blocking serum complement activity for the remainder of the extension study (Figure 1B).

Measures of hemolysis

Lactate dehydrogenase (LDH) is a standard biochemical measure of intravascular hemolysis, and levels are frequently elevated in patients with PNH.[1-3] The immediate reduction in LDH levels observed in all patients during the acute-phase study was maintained during the extension study (Figure 2). Two patients demonstrated transient increases in LDH levels (Figure 2, lines 1-2) that correlated temporally with the breakthroughs in serum complement activity shown in Figure 1. As complement blockade was restored, LDH levels returned to near-normal values.

Levels of various markers of hemolysis and platelet counts during eculizumab treatment are shown in Table 1. LDH levels decreased from a mean of 3110.7 ± 598.4 IU/L during the 52 weeks before treatment to 594.0 ± 31.7 IU/L and 622.4 ± 41.1 IU/L (normal range at Leeds Teaching Hospitals, 150-480 IU/L) during the 12 and 64 weeks of treatment, respectively ($P = .002$ for 64-week comparison in all patients). Similarly, aspartate aminotransferase (AST) levels, another marker of hemolysis, decreased from a mean baseline value of 76.2 ± 16.0 IU/L to 26.2 ± 2.3 IU/L and 30.1 ± 3.2 IU/L (normal range at Leeds Teaching Hospitals, 10-40 IU/L) during the 12 and 64 weeks of treatment, respectively ($P = .02$ for 64-week comparison in all patients). The dramatic reduction in hemolysis during eculizumab treatment was demonstrated in both noncytopenic (platelet count ≥ 150×10^9/L) and cytopenic (platelet count < 150×10^9/L) patient populations. Levels of haptoglobin, hemoglobin, and bilirubin and numbers of reticulocytes and platelets did not change significantly in the comparison of prestudy values and 64-week treatment values.

Proportions of PNH blood cells

Clonal expansion of hematopoietic stem cells with a reduction in or an absence of GPI-linked membrane proteins (type II or type III cells, respectively) is a hallmark of PNH. The effect of eculizumab on the proportions of various PNH blood-cell types was assessed (Table 2). The proportion of PNH type III RBCs relative to the total RBC population increased from 36.7% ± 5.9% at baseline to 59.2% ± 8.0% and 58.4% ± 8.5% during the 12 and 64 weeks of treatment, respectively ($P = .005$ for 64-week comparison in all patients). The increase in the proportion of PNH type III RBCs was more pronounced in patients without cytopenia than in those with cytopenia. However, the lower proportion of type III RBCs in patients with cytopenia was a direct result of the dilutional effect from the increased administration of normal RBCs because of the higher transfusion requirement in this patient group. The proportion of PNH type II RBCs increased from 5.3% ± 1.4% before treatment to 13.2% ± 2.4% ($P = .013$) during the 64 weeks of treatment. Mean proportions of type III neutrophils and platelets were greater than 90% before eculizumab therapy and were stable during the study.

Paroxysm and transfusion rates

The presence of hemoglobin in the urine (hemoglobinuria) is characteristic of PNH and a central component of periodic exacerbations known as paroxysms. In the eculizumab study, paroxysm rates were defined as the number of days with the presence of gross hemoglobinuria, defined as a colorimetric urine score of 6 or greater on a scale of 1 to 10.[2] In patients whose urine scores were assessed, the paroxysm rate decreased from 3.0 paroxysms per patient each month before eculizumab treatment to 0.1 paroxysms per patient each month during the initial 12 weeks and 0.2 paroxysms per patient each month during the total 64 weeks of treatment (Figure 3; $P < .001$).

During the extension study, 2 patients did not sustain levels of eculizumab necessary to consistently block complement (Figure 1). This breakthrough in serum hemolytic activity occurred in the last 2 days of the 14-day dosing interval, a pattern that was repeated between multiple doses. A detailed analysis of the dosing interval between visits 4 and 5 in one of the breakthrough patients showed that sufficient levels of eculizumab (PK ≥ 35 μg/mL) were present to completely block serum hemolytic activity (PD ≤ 20% hemolytic activity) for the first 12 days (Figure 4). Effective complement blockade during these 12 days was reflected by normal urine color scores and low LDH and AST levels. On the 13th and 14th days of the dosing interval, a paroxysm occurred, as evidenced by severe hemoglobinuria (black urine), dysphagia, and dramatic increases in LDH and AST levels. These events correlated with insufficient levels of eculizumab (PK) and the return of serum hemolytic activity (PD). The patient was again dosed on day 14, which resulted in the resolution of hemoglobinuria and dysphagia by the next morning. A reduction in the dosing interval from 14 to 12 days between visits 5 and 9 was sufficient to maintain the levels of

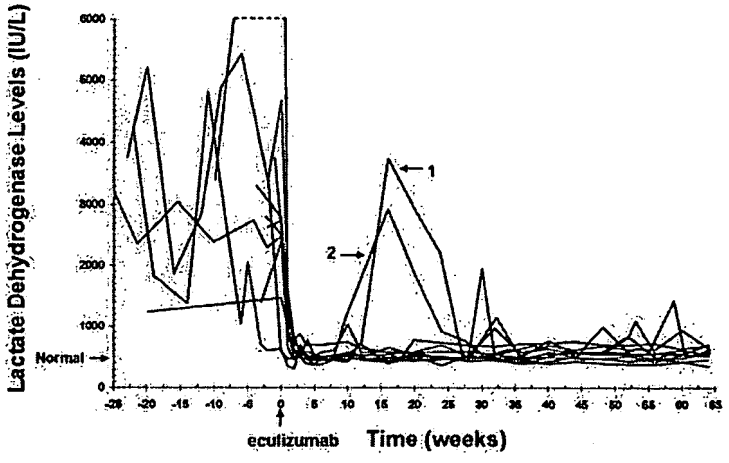


Figure 2. LDH levels in patients with PNH before and during eculizumab treatment. Initiation of eculizumab dosing is indicated at time 0 on the x axis. LDH values are shown for 11 patients with PNH for 25 weeks before and 64 weeks during eculizumab treatment. (Normal) Upper limit of normal of the LDH range at the Leeds Teaching Hospital. The dashed line indicates off-scale points from one patient with a peak value of 12 100 IU/L. Two patients who experienced a return of serum hemolytic activity during treatment are identified (patients 1 and 2).

Table 1. Changes in levels of various markers of hemolysis and platelet counts during eculizumab therapy

Biochemical marker	Normal range	Time of analysis‖ Before study†	12 wk	64 wk	P*
LDH, IU/L					
All patients	150-480	3110.7 ± 598.4	594.0 ± 31.7	622.4 ± 41.1	.002
Patients without cytopenia‡	NA	3965.0 ± 971.7	617.2 ± 24.3	657.1 ± 54.8	—
Patients with cytopenia§	NA	2085.0 ± 267.0	566.3 ± 65.5	580.8 ± 63.2	—
AST, IU/L					
All patients	10-40	76.2 ± 16.0	26.2 ± 2.3	30.1 ± 3.2	.02
Patients without cytopenia	NA	88.5 ± 28.3	24.4 ± 3.8	30.8 ± 5.7	—
Patients with cytopenia	NA	61.4 ± 9.8	28.4 ± 2.4	29.1 ± 2.3	—
Haptoglobin, g/L					
All patients	0.5-2	0.06 ± 0	0.07 ± .01	0.14 ± 0.07	NS
Patients without cytopenia	NA	0.06 ± 0	0.08 ± .02	0.20 ± 0.12	—
Patients with cytopenia	NA	0.06 ± 0	0.06 ± 0	0.08 ± 0.01	—
Hemoglobin, g/dL					
All patients	11.5-18	10.0 ± 0.4	10.3 ± 0.4	10.4 ± 0.4	NS
Patients without cytopenia	NA	10.4 ± 0.5	10.9 ± 0.6	10.8 ± 0.5	—
Patients with cytopenia	NA	9.5 ± 0.7	9.6 ± 0.4	9.9 ± 0.5	—
Bilirubin, μM					
All patients	3-15	25.9 ± 4.3	28.2 ± 4.4	28.7 ± 4.0	NS
Patients without cytopenia	NA	30.6 ± 7.4	34.9 ± 6.6	34.6 ± 5.9	—
Patients with cytopenia	NA	20.2 ± 2.2	20.2 ± 3.6	21.7 ± 3.6	—
Reticulocytes, $\times 10^{-3}/mm^3$					
All patients	20-80	161.4 ± 25.9	191.2 ± 23.6	189.6 ± 21.8	NS
Patients without cytopenia	NA	200.2 ± 38.4	243.4 ± 26.2	233.2 ± 27.4	—
Patients with cytopenia	NA	114.8 ± 21.9	128.5 ± 15.6	137.3 ± 15.2	—
Platelets, $\times 10^9/L$					
All patients	150-400	183.0 ± 35.3	183.6 ± 37.8	180.8 ± 35.8	NS
Patients without cytopenia	NA	250.2 ± 49.8	256.3 ± 51.8	251.5 ± 47.2	—
Patients with cytopenia	NA	102.2 ± 11.8	96.3 ± 18.7	95.9 ± 19.7	—

— indicates not determined; NA, not applicable; and NS, not significant.

*Comparisons of mean change from the prestudy period to the 64-week treatment period for all patients.

†Mean values during 52-week period before treatment except for AST, which represents the baseline mean.

‡Platelet count ≥ 150 × 10^9/L; n = 6.

§Platelet count < 150 × 10^9/L; n = 5.

‖Values are presented as ± standard error (SE).

Table 2. Changes in proportions of PNH blood-cell types during eculizumab treatment

PNH cell type	Proportion of PNH cells, % Baseline§	12 wk§	64 wk§	P*
Type III RBCs				
All patients	36.7 ± 5.9	59.2 ± 8.0	58.4 ± 8.5	.005
Patients without cytopenia†	38.8 ± 3.7	73.5 ± 4.1	67.8 ± 7.0	—
Patients with cytopenia‡	34.2 ± 12.9	42.1 ± 13.8	47.0 ± 16.3	—
Type II RBCs				
All patients	5.3 ± 1.4	7.5 ± 2.1	13.2 ± 2.4	.013
Patients without cytopenia	4.8 ± 1.8	6.8 ± 3.0	12.9 ± 1.9	—
Patients with cytopenia	5.8 ± 2.4	8.4 ± 3.2	13.5 ± 5.0	—
Type III WBCs				
All patients	92.1 ± 4.6	89.9 ± 6.6	91.1 ± 5.8	NS
Patients without cytopenia	95.1 ± 2.3	94.9 ± 2.6	96.1 ± 1.1	—
Patients with cytopenia	88.4 ± 10.2	83.9 ± 14.5	85.2 ± 12.9	—
Type III platelets				
All patients	92.4 ± 2.4	93.3 ± 2.8	92.8 ± 2.6	NS
Patients without cytopenia	93.2 ± 1.4	95.8 ± 2.1	95.0 ± 0.8	—
Patients with cytopenia	91.6 ± 5.4	90.2 ± 5.6	90.2 ± 5.8	—

— indicates not determined; WBCs, white blood cells; and NS, not significant.

*Comparisons of mean change from baseline to the 64-week treatment period for all patients.

†Platelet count ≥ 150 × 10^9/L; n = 6.

‡Platelet count < less than 150 × 10^9/L; n = 5.

§Data are presented as ± SE.

eculizumab necessary to effectively and consistently block serum hemolytic activity and, therefore, intravascular hemolysis in these patients (Figure 4, visit 9).

A statistically significant reduction in the rate of packed red blood cell (PBRC) transfusion was observed in the acute-phase study and was maintained during the extension study compared with the rate of transfusion before eculizumab therapy (Table 3). Mean transfusion rates decreased from 2.1 U per patient each month during the 1-year period before treatment to 0.6 U per patient each month during the initial 12-week and to 0.5 U per patient each month during the total 64-week treatment period.

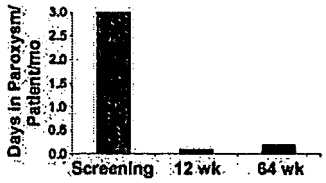
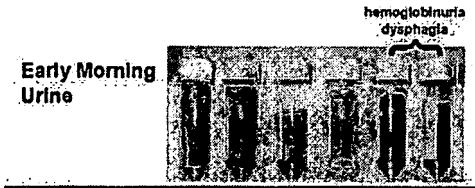


Figure 3. Paroxysm rate in patients with PNH before and during eculizumab treatment. A urine color scale[2] was used to assess the incidence of paroxysms in 8 patients with PNH before and during treatment with eculizumab. Paroxysm was prospectively defined by a urine colorimetric score of 6 or more. Bars represent the paroxysm rates (number of paroxysms per patient per month) during the screening period, during the first 12 weeks, and over the total 64 weeks of eculizumab treatment. Three patients were not included in the analysis either because their pretreatment urine scores were inadvertently not collected (2 patients) or because an iron-chelating agent that resulted in artificially colored urine was administered during the extension study (1 patient).



Day (visit)	0*(4)	1-8	9	10	11	12	13	0*(5)	1-2	0*(9)
Urine	10	2-3	3	3	3	3	9	10	3	2
LDH	2624	—	784	—	—	697	1687	2917	—	495
AST	119	—	38	—	—	31	87	—	—	—
PK	28	—	51	—	—	35	31	23	—	51
PD	72	—	1	—	—	2	56	56	—	2

Figure 4. Relationship between complement inhibition with eculizumab and various hemolytic parameters and symptoms during a transient breakthrough in serum-complement activity. Urine color, symptoms, biochemical parameters of hemolysis, PK, and PD were assessed during a 14-day eculizumab-dosing interval in a patient with a transient breakthrough in serum hemolytic activity. Eculizumab was administered on day 0 after assay samples were collected. A urine colorimetric score of 6 or greater was considered abnormal (hemoglobinuria). Levels of the hemolytic markers LDH and AST are shown (IU/L). Eculizumab serum concentrations (PK, μg/mL) of ≥ 35 μg/mL were sufficient to maintain a serum hemolytic activity (PD, % serum hemolytic activity) of ≤ 20%, a value known to represent complete complement blockade. The eculizumab-dosing interval was reduced to 12 days between visits 5 and 9. Urine row presents colorimetric scores. — indicates not determined. *Dose of eculizumab.

Similarly, median transfusion rates decreased from 1.8 U per patient each month before treatment to 0.0 U and 0.3 U per patient each month during the first 12 weeks and the total 64 weeks of therapy, respectively ($P = .001$ for 64-week comparison in all patients). The reduction in transfusions was more pronounced in patients without cytopenia; the mean rate in this population decreased from 2.4 U per patient each month during the prestudy period to 0.2 U per patient each month during the 12- and 64-week treatment periods. Transfusion reduction in patients with poor bone marrow reserve (patients with cytopenia) decreased from a mean rate of 1.8 U per patient each month during the 1-year pretreatment period to 1.2 and 0.8 U per patient each month during the 12- and 64-week treatment periods, respectively.

Quality-of-life measurements

Quality-of-life measurements were assessed using the European Organization for Research and Treatment of Cancer (EORTC) QLQ-C30 instrument during the total 64-week treatment period and were compared with baseline values (Table 4). Significant improvements in quality-of-life domains that were demonstrated in the acute-phase study were maintained in the extension study, including global health status ($P = .009$), physical functioning ($P < .001$), emotional functioning ($P < .001$), cognitive functioning ($P = .001$), fatigue ($P < .001$), dyspnea ($P < .001$), and insomnia ($P = .031$). In addition, changes in role functioning ($P = .003$) and pain domains ($P = .023$) achieved significance during the 64-week treatment period ($P < .001$). The constipation domain showed a significant increase over the treatment period. Taken together, these results indicate that various parameters of quality of life in patients with PNH rapidly improve with eculizumab therapy and that these changes are maintained for extended lengths of time.

Table 4. Quality-of-life assessment during eculizumab treatment

Domain*	Mean baseline score†	64-wk change from baseline score‡	P§
Global health status	56.1	13.8	.009
Physical functioning	70.9	14.3	<.001
Emotional functioning	70.5	12.6	<.001
Role functioning	66.7	14.5	.003
Cognitive functioning	77.3	10.3	.001
Fatigue	47.5	−17.8	<.001
Dyspnea	39.4	−16.6	<.001
Insomnia	30.3	−8.2	.031
Pain	21.2	−8.2	.023
Constipation	3.0	4.1	<.001

*Quality of life was assessed using the European Organization for Research and Treatment of Cancer QLQ-C30 instrument.

†Mean values of linearly transformed scores.

‡Values represent least-square means. Positive change indicates improvement on Global Health Status and Functional scales, and negative change indicates improvement on Symptom scales.

§From a mixed analysis-of-covariance model with visit as a fixed effect, patient as a random effect, and baseline as a covariate.

Safety

All patients completed the extension study. No deaths occurred and no thromboses developed during the 12-month treatment period. In no patients were antibodies against eculizumab detected. All patients had at least one adverse event (AE), and one patient had a serious adverse event (SAE). No patient withdrew because of an AE. The most common AEs were flulike symptoms (4 patients), sore throat (4 patients), pain (3 patients), nausea (3 patients), bruising (3 patients), cough (3 patients), and upper respiratory infection (3 patients).

One patient experienced an SAE during the extension study characterized by neutropenia with extravascular hemolysis, which was thought to have been caused by a viral syndrome. Briefly, the patient had a 5-year history of PNH with aplastic anemia (with a normal neutrophil count) and had been on eculizumab therapy for 14 months. A viral syndrome developed, and during the next several days hemoglobin levels dropped from 10.2 to 5.3 g/dL, whereas serum LDH levels increased only slightly. The patient

Table 3. Changes in transfusion requirements during eculizumab treatment

Patient group	Transfusion rates: Before study* Mean	Before study* Median	12 wk Mean	12 wk Median	64 wk Mean	64 wk Median	P†
All patients	2.1	1.8	0.6	0.0	0.5	0.3	.001
Patients without cytopenia‡	2.4	2.0	0.2	0.0	0.2	0.1	–
Patients with cytopenia§	1.8	1.8	1.2	0.7	0.8	0.7	–

Transfusion rates are units per patient per month.

– indicates not determined.

*Values during 52-week period before treatment.

†Comparisons of median change from before study to the 64-week treatment period.

‡Platelet count ≥ 150 × 10^9/L; n = 6.

§Platelet count < 150 × 10^9/L; n = 5.

received a transfusion of 4 U PBRCs; at the next study visit, the hemoglobin level had returned to 10.7 g/dL and the platelet count was normal, but the neutrophil count was abnormally low (260/μL). Two weeks later, the neutrophil count had recovered to almost-normal levels (1600/μL). The investigator did not think the SAE was caused by the study medication. The patient remained transfusion independent for the remainder of the extension study.

Discussion

We reported previously that eculizumab therapy in patients with PNH resulted in a dramatic reduction in hemolysis and transfusion requirements. The extension study was conducted to obtain long-term data regarding eculizumab use in this patient population. Here we report the sustained efficacy and safety of eculizumab in a 1-year, open-label extension study.

Serum hemolytic activity in 9 of 11 patients was completely blocked throughout the 64-week treatment period, with trough levels of eculizumab at equilibrium ranging from approximately 35 μg/mL to 350 μg/mL. One patient had a breakthrough of serum hemolytic activity during the acute-phase study, and another patient broke through early in the extension study. PK analysis of both breakthrough patients demonstrated a simultaneous decrease in eculizumab level below that required to completely block complement activity (≥ 35 μg/mL).[9] Adjustment of the eculizumab dosing interval in these 2 patients from every 14 days to every 12 days successfully sustained trough levels of eculizumab higher than 35 μg/mL and consistently blocked serum hemolytic activity for the remainder of the extension study. The effective and consistent blockade of complement achieved with the 12-day dosing interval was supported by the resolution of symptoms, including hemoglobinuria and dysphagia, and lower levels of LDH and AST (Figure 4). Taken together, these data illustrate the tight relationship between complement blockade, hemolysis, and symptoms in PNH.

The dramatic reduction in LDH levels that was demonstrated immediately on the administration of eculizumab in the initial study was sustained during the extension study, providing strong evidence that eculizumab effectively and durably inhibits intravascular hemolysis in patients with PNH. LDH levels were reduced from a mean of 3110.7 IU/L during the 52-week period before treatment to a mean of 622.4 IU/L during the 64 weeks of eculizumab therapy. In addition, detailed analysis of PK and PD demonstrated a strong correlation between the return of complement activity, hemolysis, and an increase in LDH levels (Figure 4). These data confirm that LDH levels can be used as an accurate measure of intravascular hemolysis in PNH, and they provide evidence that effective complement blockade during eculizumab therapy can be determined by monitoring levels of this enzyme.

LDH levels remained slightly elevated in most patients during eculizumab treatment, suggesting low levels of ongoing hemolysis. Undetectable haptoglobin levels and elevated bilirubin levels also support residual hemolysis in the midst of terminal complement inhibition. This may be attributed to yet undefined, noncomplement-mediated mechanisms of PNH RBC clearance, as recently described.[11] Alternatively, it is possible that a fraction of PNH RBCs is cleared through complement-mediated events before C5, such as a C3b coating and clearance. In this regard, slightly elevated levels of LDH have also been reported in a patient with coexistent PNH and C9 deficiency.[12]

The marked reduction in transfusions demonstrated in the initial eculizumab study was sustained in the extension study. Further, patients who had good bone marrow reserve (those without cytopenia) showed greater reductions in transfusion requirements than did patients who had hypoplasia (those with cytopenia). Importantly, 3 patients with hypoplasia who received transfusions during the acute-phase study were subsequently treated with erythropoietin during the extension study. Two of the 3 patients responded with an increase in reticulocyte counts and a further reduction in transfusion requirements (data not shown). These data suggest that erythropoietin therapy in PNH patients with hypoplasia increases erythropoiesis and that concomitant eculizumab therapy protects against increased hemolysis, with a resultant improvement in response. Three of 11 PNH patients remain transfusion independent after 64 weeks of eculizumab therapy.

Interestingly, eculizumab therapy resulted in dramatic reductions in transfusion requirements even though hemoglobin levels and reticulocyte counts frequently remained fairly constant. However, before eculizumab treatment, hemoglobin levels in patients were artificially maintained through regular transfusions of packed RBCs. Thus, stabilization of hemoglobin levels with a concomitant reduction in or cessation of transfusions likely represents a net increase in hemoglobin levels. These data suggest that the resolution of hemolysis in patients with PNH results in a new steady state hemoglobin level, determined by a combination of the extent of the underlying bone marrow dysfunction, the size of the type III RBC clone, and the new level (if any) of transfusion requirement.

There was a sustained improvement in quality of life for transfusion-dependent PNH patients administered eculizumab, as measured by the EORTC QLQ-C30. Quality-of-life parameters that showed significant improvement include global health status, physical functioning, emotional functioning, role functioning, cognitive functioning, fatigue, dyspnea, insomnia, and pain. Improvements in quality-of-life parameters in PNH patients taking eculizumab will be further investigated in subsequent placebo-controlled trials.

Clinical assessment of PNH symptoms not captured by the QLQ-C30 instrument, such as dysphagia, abdominal pain, and erectile failure, showed complete resolution, or at least dramatic improvement, during eculizumab treatment.[13] These symptoms have been shown to correlate with a large PNH type III clone size, suggesting that they are related to excessive hemolysis.[14] In addition, these symptoms have been attributed to smooth muscle dystonia caused by the scavenging of nitric oxide by free plasma hemoglobin.[14-18] The capacity of free plasma hemoglobin to scavenge nitric oxide during intravascular hemolysis has been demonstrated in patients with sickle cell anemia.[19] The relationship between levels of free plasma hemoglobin and nitric oxide in patients with PNH remains to be elucidated.

Thrombosis accounts for most deaths in patients with PNH. Studies have reported a strong correlation between a large PNH type III neutrophil clone and the occurrence of thrombosis.[14,20] Hall et al[20] reported that in approximately 44% of patients with large PNH clones, venous thrombosis developed in the first 10 years after diagnosis. The tendency toward thrombosis in patients with PNH is thought to be multifactorial in etiology, involving both the absence of GPI-anchored complement inhibitors on the surfaces of circulating platelets and the high levels of intravascular free plasma hemoglobin with the consequent scavenging of nitric oxide.[18,21-25] It is reasonable to hypothesize that thrombosis resulting from either or both of these mechanisms should be reduced by terminal complement blockade. Although no thrombotic events occurred

during eculizumab treatment, 6 of the 11 patients were on warfarin therapy before and during the study. Further analyses and studies are required to investigate whether eculizumab reduces the risk for thrombosis in patients with PNH.

Eculizumab continued to be safe and well tolerated in the PNH extension study. No deaths occurred and no thromboses developed in this study. Each patient had at least one AE; flulike symptoms and sore throat were the most common. Evaluation of AEs in other placebo-controlled eculizumab studies suggests that the reported AEs in the PNH extension study were similar in type and frequency to those reported in other placebo treatment groups. The single SAE, transient neutropenia, was not thought by the principal investigator to be related to study medication; rather, it was thought to be the result of a viral syndrome. All patients chose to continue treatment in a second extension study, and evaluation of eculizumab therapy in a broader population of PNH patients is under way.

Results of this 1-year extension study showed that eculizumab therapy continues to be safe and well tolerated in PNH patients. Additionally, long-term complement inhibition with eculizumab in this patient population has resulted in sustained reductions in hemolysis and blood transfusions and continued improvement in quality of life.

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ORIGINAL ARTICLE

The Complement Inhibitor Eculizumab in Paroxysmal Nocturnal Hemoglobinuria

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ABSTRACT

BACKGROUND

We tested the safety and efficacy of eculizumab, a humanized monoclonal antibody against terminal complement protein C5 that inhibits terminal complement activation, in patients with paroxysmal nocturnal hemoglobinuria (PNH).

METHODS

We conducted a double-blind, randomized, placebo-controlled, multicenter, phase 3 trial. Patients received either placebo or eculizumab intravenously; eculizumab was given at a dose of 600 mg weekly for 4 weeks, followed 1 week later by a 900-mg dose and then 900 mg every other week through week 26. The two primary end points were the stabilization of hemoglobin levels and the number of units of packed red cells transfused. Biochemical indicators of intravascular hemolysis and the patients' quality of life were also assessed.

RESULTS

Eighty-seven patients underwent randomization. Stabilization of hemoglobin levels in the absence of transfusions was achieved in 49% (21 of 43) of the patients assigned to eculizumab and none (0 of 44) of those assigned to placebo ($P<0.001$). During the study, a median of 0 units of packed red cells was administered in the eculizumab group, as compared with 10 units in the placebo group ($P<0.001$). Eculizumab reduced intravascular hemolysis, as shown by the 85.8% lower median area under the curve for lactate dehydrogenase plotted against time (in days) in the eculizumab group, as compared with the placebo group (58,587 vs. 411,822 U per liter; $P<0.001$). Clinically significant improvements were also found in the quality of life, as measured by scores on the Functional Assessment of Chronic Illness Therapy-Fatigue instrument ($P<0.001$) and the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire. Of the 87 patients, 4 in the eculizumab group and 9 in the placebo group had serious adverse events, none of which were considered to be treatment-related; all these patients recovered without sequelae.

CONCLUSIONS

Eculizumab is an effective therapy for PNH. (ClinicalTrials.gov number, NCT00122330.)

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Exhibit G

PAROXYSMAL NOCTURNAL HEMOGLOBINuria (PNH), an uncommon form of hemolytic anemia, results from the clonal expansion of hematopoietic stem cells that have somatic mutations in the X-linked gene *PIG-A*.[1,2] *PIG-A* mutations cause an early block in the synthesis of glycosylphosphatidylinositol (GPI) anchors, which tether many proteins to the cell surface. Consequently, the blood cells in patients with PNH have a partial deficiency (type II) or a complete deficiency (type III) of GPI-linked proteins.

Intravascular hemolysis is a prominent feature of PNH and is the consequence of the absence of the GPI-linked complement regulatory protein CD59.[3,4] CD59 blocks the formation of the terminal complement complex (also called the membrane-attack complex) on the cell surface, thereby preventing erythrocyte lysis and in vitro platelet activation.[5-8] Excessive or persistent intravascular hemolysis in patients with PNH causes anemia, hemoglobinuria, and complications related to the presence of plasma free hemoglobin, including thrombosis, abdominal pain, dysphagia, erectile dysfunction, and pulmonary hypertension.[9-12] Indeed, the symptoms in PNH are often disproportionate to the degree of anemia. Many patients with this disease are dependent on transfusions. Currently, there is no therapy that effectively reduces intravascular hemolysis or improves the symptoms in patients with PNH.

Eculizumab (Soliris, Alexion Pharmaceuticals) is a humanized monoclonal antibody directed against the terminal complement protein C5.[13] In a preliminary, 12-week, open-label clinical study involving 11 patients with PNH, eculizumab reduced intravascular hemolysis and the patients' transfusion requirements.[14] However, this two-center, uncontrolled study did not have a control group or predefined criteria for the administration of a transfusion, such as a predefined hemoglobin level at which transfusions were administered or a prespecified number of units of packed red cells for a given hemoglobin level.

We report the results of the phase 3 Transfusion Reduction Efficacy and Safety Clinical Investigation, a Randomized, Multicenter, Double-Blind, Placebo-Controlled, Using Eculizumab in Paroxysmal Nocturnal Hemoglobinuria (TRIUMPH) study, which investigated whether eculizumab stabilized hemoglobin levels and reduced transfusion requirements in 87 transfusion-dependent patients with PNH during 6 months of treatment. Intravascular hemolysis and the quality of life were also assessed.

METHODS

PATIENTS

The trial consisted of a 2-week screening period, an observation period of up to 3 months, and a 26-week treatment period. Patients 18 years of age or older who had received at least four transfusions during the previous 12 months were eligible. A PNH type III erythrocyte proportion of 10% or more, platelet counts of at least 100,000 per cubic millimeter, and lactate dehydrogenase levels that were at least 1.5 times the upper limit of the normal range were also required. Concomitant administration of erythropoietin, immunosuppressive drugs, corticosteroids, coumarins, low-molecular-weight heparins, iron supplements, and folic acid were permitted, provided that the doses were constant before and throughout the study. Because persons who have a genetic deficiency of terminal complement proteins have an increased risk of neisserial infections, all patients were vaccinated against *Neisseria meningitidis* with the use of locally approved vaccines. The protocol was approved by the institutional review board at each center, and all patients gave written informed consent.

Patients receiving transfusions who had a mean hemoglobin level greater than 10.5 g per deciliter before transfusion during the 12 months before entry into the study or who had received another investigational drug within 30 days before the first visit were excluded. Patients who had a complement deficiency, an active bacterial infection, or a history of meningococcal disease and those who had undergone bone marrow transplantation were also excluded.

An individualized transfusion algorithm was calculated for each patient on the basis of the history of transfusions during the previous 12 months; the written algorithm documented the number of units of packed red cells to be transfused for given hemoglobin values and served as a prospectively determined guide for transfusion during the observation and treatment periods. Before randomization, eligible patients were observed for up to 13 weeks. Patients who did not require a transfusion during the observation period were considered ineligible. A transfusion administered to a patient who had a hemoglobin

level of 9 g per deciliter or less with symptoms or 7 g per deciliter or less with or without symptoms qualified the patient for the study (qualifying transfusion) and established the hemoglobin set point. This set point was required for the primary efficacy variable and was individualized for each patient.

STUDY DESIGN

Randomization was performed centrally in a 1:1 ratio without blocking and with stratification according to the number of units of packed red cells transfused during the past year; patients were assigned, by means of an interactive voice–response system, to receive either placebo or eculizumab within 10 days after the administration of the qualifying transfusion. Patients received infusions of 600 mg of eculizumab or placebo every week (±2 days) for 4 weeks, followed 1 week (±2 days) later by 900 mg of eculizumab or placebo, and then by a maintenance dose of 900 mg of eculizumab or placebo every 2 weeks (±2 days) through week 26.

CLINICAL EFFICACY

The two primary end points were the stabilization of hemoglobin levels, defined as a hemoglobin value that was maintained above the level at which the qualifying transfusion was administered, in the absence of transfusions during the 26-week treatment period, and the number of units of packed red cells transfused during that period. The trigger for the administration of transfusions during the study remained unchanged: patients received transfusions when they had symptoms resulting from anemia and their hemoglobin levels reached the individualized, predetermined set point. Prespecified secondary end points included transfusion independence; hemolysis, as measured by the lactate dehydrogenase value for the area under the curve from baseline to 26 weeks; and changes in the level of fatigue, as assessed from baseline to 26 weeks with the use of the Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-Fatigue) instrument (scores can range from 0 to 52, with higher scores indicating improvement in fatigue).[15] Prespecified exploratory analyses included assessment of the quality of life with the use of the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire (EORTC QLQ-C30) (scores can range from 0 to 100, with higher scores on the global health status and functioning scales and lower scores on the symptom scales and single-item measures indicating improvement)[16]; changes in lactate dehydrogenase levels from baseline through week 26; and the presence of thrombosis. Other prespecified measurements included the pharmacokinetics, pharmacodynamics, and immunogenicity of eculizumab. The time to the first transfusion during the treatment period and the proportion of PNH type III blood cells were assessed.

SAFETY

Adverse events related to study infusions and vital signs (assessed at each of the 17 study visits during treatment), the results of biochemical analyses and blood counts (assessed at 9 visits), and findings on electrocardiograms (assessed at 3 visits) were documented. Adverse events were coded with the use of preferred terms from the Medical Dictionary for Regulatory Activities (MedDRA) (www.msso.org/MSSOWeb/index.htm) and tabulated as incidence rates in the two study groups.

STATISTICAL ANALYSIS

The planned sample size of 75 patients provided the study with a statistical power of 82%, at an alpha level of 0.05, to detect an increase of 35 percentage points (i.e., a change from 20% to 55%) in the rate of the stabilization of hemoglobin levels and a reduction in the median number of units of packed red cells transfused from 6 to 2 (±2). For the two primary end points, the analyses were performed according to the intention-to-treat principle with the use of data on all 87 patients who underwent randomization; stabilization of hemoglobin levels was analyzed with the use of Fisher's exact test, and the total number of units of packed red cells transfused was analyzed with the use of the Wilcoxon rank-sum test. To assess the effect of treatment on whether or not transfusions were required, Fisher's exact test was used. The log-rank test was used to compare the time to the first transfusion in the two groups. The area under the curve for lactate dehydrogenase was compared between the two groups with the use of the Wilcoxon rank-sum test.

Fatigue was assessed according to the scoring guidelines for the FACIT-Fatigue instrument.[17] The assessment of the quality of life was based on the EORTC QLQ-C30 scores and was conducted as described previously.[18] Changes in scores

on the FACIT-Fatigue instrument and the EORTC QLQ-C30 instrument from baseline through week 26 were analyzed with the use of a mixed model, with baseline scores as the covariate, treatment and time as fixed effects, and the patient identifier as a random effect. Changes in the levels of lactate dehydrogenase, PNH type III erythrocytes, and hemoglobin from baseline through week 26 were analyzed with the use of the same mixed model. All reported P values are two-sided and were not adjusted for multiple analyses. The incidence rates of adverse events were compared with the use of Fisher's exact test. No interim analyses were performed, and blinding regarding the results was maintained until the end of the study.

The authors and the sponsor were jointly responsible for the trial design and the development of the protocol. Data were collected by an electronic case-report form with the use of InForm software (version 4.0, Phase Forward) and were analyzed by the sponsor. The decision to publish the trial data and final decisions on the content of the manuscript rested with Dr. Hillmen in consultation with the other authors. The manuscript was prepared by Dr. Hillmen, with substantial review and comments by the other authors. All authors had access to the primary data and take responsibility for the veracity and completeness of the data reported.

Table 1. Baseline Characteristics of the Patients.

Characteristic	Placebo Group (N=44)	Eculizumab Group (N=43)
Sex — no.		
Male	15	20
Female	29	23
Age — yr		
Median	35	41
Range	18–78	20–85
Duration of PNH — yr		
Median	9.2	4.3
Range	0.5–38.5	0.9–29.8
Reticulocyte counts — per mm³		
Median	204,400	206,600
Range	45,400–556,200	40,200–570,400
History of aplastic anemia — no. (%)	12 (27)	6 (14)
History of myelodysplastic syndrome — no. (%)	0	2 (5)
History of thrombosis — no. (%)	8 (18)	9 (21)
Total no. of thrombotic events	11	16
Use of erythropoietin — no. (%)	0	3 (7)
Use of cyclosporine — no. (%)	1 (2)	1 (2)
Use of anticoagulant agents (coumarins or heparins) — no. (%)	11 (25)	21 (49)
Use of corticosteroids or androgenic steroids — no. (%)	12 (27)	12 (28)

RESULTS

PATIENTS' CHARACTERISTICS

Of a total of 115 patients with PNH who underwent screening, 87 (35 men and 52 women) at 34 sites in the United States, Canada, Europe, and Australia who received a qualifying transfusion, met the inclusion criteria and did not meet any of the exclusion criteria were randomly assigned to eculizumab (43 patients) or placebo (44 patients) between October 2004 and June 2005. At each of 16 sites one patient underwent randomization, at each of 6 sites two patients underwent randomization, and at each of 12 sites 3 or more patients underwent randomization. There were no significant differences in the baseline characteristics of the patients in the two groups (Table 1).

Of 87 patients who underwent randomization, 85 completed the trial (see the Supplementary Appendix, available with the full text of this article at www.nejm.org). Two patients in the eculizumab group did not complete the trial, one because traveling to the study center was inconvenient and the other because of pregnancy; these patients were included in the analyses. Ten patients in the placebo group discontinued infusions because of a perceived lack of efficacy but remained in the study for monitoring, as prespecified in the protocol, and were included in the analyses.

PHARMACOKINETICS AND PHARMACODYNAMICS

In 42 of 43 patients in the eculizumab group, a 900-mg dose of eculizumab every 2 weeks (±2 days) completely blocked serum hemolytic activity, as assessed by a presensitized erythrocyte hemolytic assay,[14] throughout the study period. In one patient, therapeutic trough levels of eculizumab were not maintained.

EFFECT ON HEMOLYSIS

The effect of eculizumab on chronic intravascular hemolysis was demonstrated by an immediate

(1 week) and sustained decrease in lactate dehydrogenase levels (Fig. 1A). In the eculizumab group, the mean (±SE) lactate dehydrogenase level decreased from 2199.7±157.7 U per liter at baseline to 327.3±67.6 U per liter at 26 weeks, whereas in the placebo group the levels remained elevated, with values of 2258.0±154.8 U per liter at baseline and 2418.9±140.3 U per liter at 26

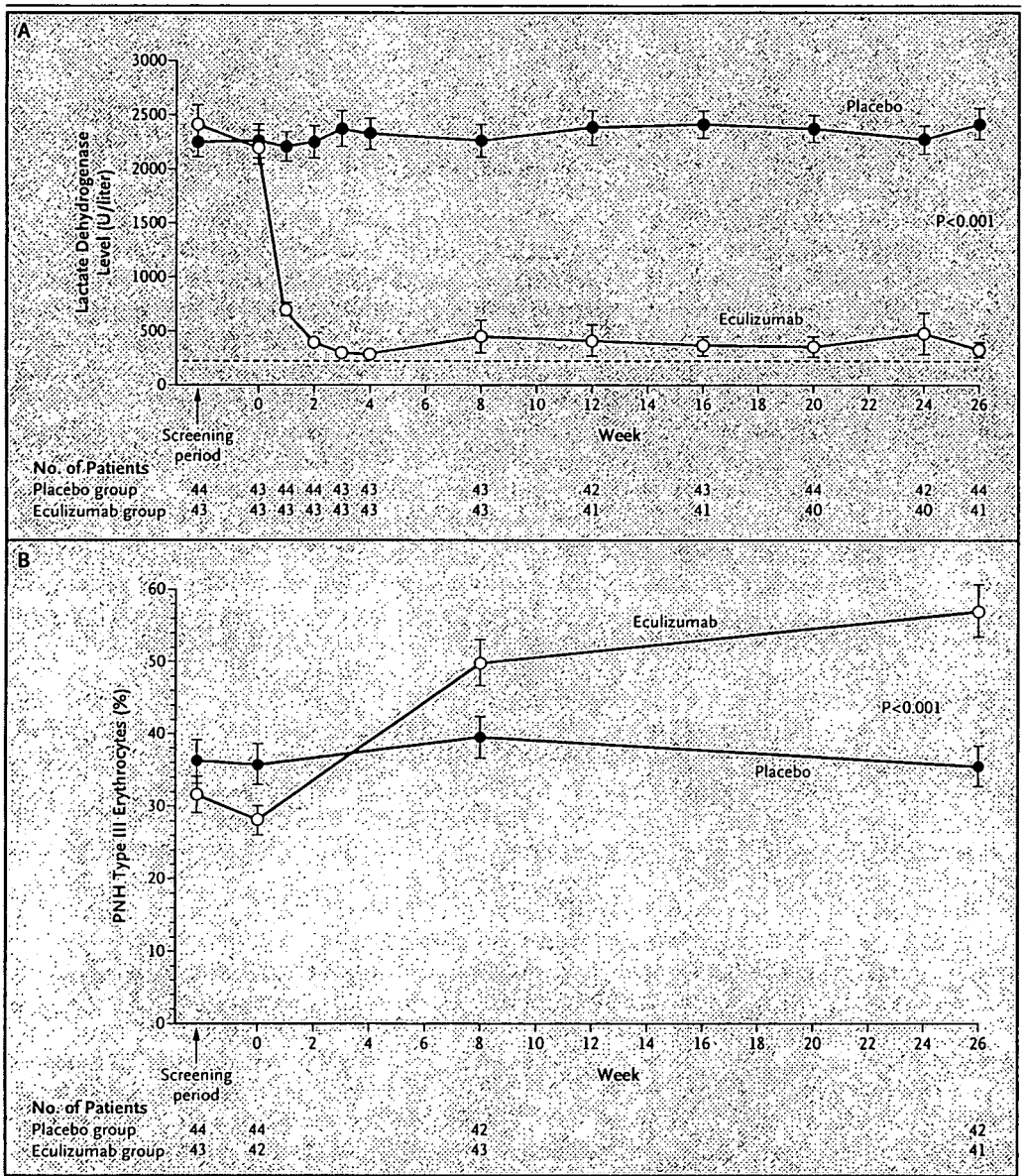

Figure 1. Levels of Lactate Dehydrogenase and PNH Type III Erythrocytes during Treatment with Eculizumab.

Panel A shows the degree of intravascular hemolysis according to the mean levels of lactate dehydrogenase from baseline (week 0) to week 26 in the two study groups. The dashed line indicates the upper limit of the normal range for lactate dehydrogenase (normal range, 103 to 223 U per liter). In the eculizumab group the mean level of lactate dehydrogenase was reduced to just above the upper limit of the normal range at week 26; of 41 patients in this group who completed the study, 15 had levels within the normal range. In the placebo group, all patients had levels at least five times above the upper limit of normal at week 26. Panel B shows the mean proportion of PNH type III erythrocytes in patients in the two groups. Screening occurred up to 3 months before week 0. P values are from a mixed analysis-of-covariance model from baseline through week 26. I bars indicate the standard error.

weeks (P<0.001). The median value of the areas under the curve for lactate dehydrogenase plotted against time (in days) was 85.8% lower in the eculizumab group than in the placebo group (58,587 vs. 411,822 U per liter; P<0.001). A second biochemical measure of hemolysis, the serum level of aspartate aminotransferase, also showed significant improvement with eculizumab, as compared with placebo (data not shown).

The reduction in intravascular hemolysis in the eculizumab group resulted in an increase in PNH type III erythrocytes (Fig. 1B) from a mean of 28.1±2.0% at baseline to 56.9±3.6% at week 26. The proportion of PNH type III erythrocytes in patients in the placebo group remained constant (35.7±2.8% before treatment and 35.5±2.8% at 26 weeks, P<0.001 for the comparison with the eculizumab group and the placebo group). The proportion of PNH type III granulocytes and monocytes did not change significantly between the two groups (data not shown).

CLINICAL EFFICACY

Primary End Points

The two primary efficacy end points were the stabilization of hemoglobin levels and the number of units of packed red cells transfused. At the end of the treatment period, 49% of patients in the eculizumab group (21 of 43) had levels of hemoglobin that remained above the prespecified set point (median, 7.7 g per deciliter for both groups) in the absence of transfusions, whereas stabilization of hemoglobin levels did not occur in any patient in the placebo group (P<0.001) (Table 2). By week 26, the median number of units of packed red cells transfused per patient was 0 in the eculizumab group and 10 in the placebo group (P<0.001), whereas the mean number of units of packed red cells transfused was 3.0±0.7 and 11.0±0.8, respectively. In the 6-month period before the study, the median number of units of packed red cells transfused per patient was 9.0 in the eculizumab cohort and 8.5 in the placebo cohort, and the mean number of units of packed red cells transfused was 9.6±0.6 and 9.7±0.7, respectively. The mean hemoglobin levels changed from 10.0±0.2 g per deciliter and 9.7±0.2 g per deciliter in the eculizumab group and the placebo group, respectively, at baseline to 10.1±0.2 g per deciliter and 8.9±0.2 g per deciliter, respectively, at week 26 (P<0.001, by mixed-model analysis).

The median time to the first transfusion was significantly longer in eculizumab-treated patients than in patients who received placebo (P<0.001) (Fig. 2). Transfusion independence was achieved in 51% of patients in the eculizumab group (22 of 43) and 0% of those in the placebo group (0 of 44, P<0.001). By week 26, the total number of units of packed red cells transfused was 131 in the eculizumab group and 482 in the placebo group (Table 2). By contrast, during the 6 months before the study, the total number of units transfused was 413 in the eculizumab group and 417 in the placebo group.

Table 2. Stabilization of Hemoglobin Levels and the Number of Units of Packed Red Cells Transfused during Treatment.*

Primary End Point	Before Treatment†		During Treatment		P Value
	Placebo Group	Eculizumab Group	Placebo Group	Eculizumab Group	
Patients with stabilized hemoglobin levels (%)	NA	NA	0	49	<0.001‡
Packed red cells transfused (units/patient)					
Median	8.5	9.0	10	0	<0.001§
Interquartile range	7–12.5	6–12	6–16	0–6	
Mean	9.7±0.7	9.6±0.6	11.0±0.8	3.0±0.7	
Total	417	413	482	131	

* Plus–minus values are means ±SE. NA denotes not applicable.

† Transfusion data obtained during 12 months before treatment were normalized to a value equivalent to the value for a 6-month period.

‡ The P value is for the comparison between groups during treatment, calculated with the use of a two-tailed Fisher's exact test.

§ The P value is for the comparison between groups during treatment, calculated with the use of the Wilcoxon rank-sum test.

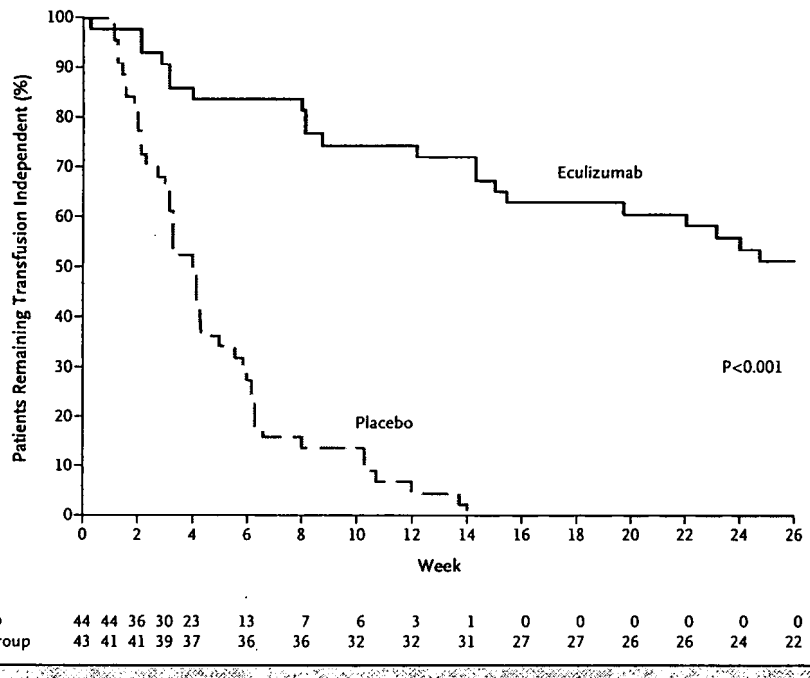

Figure 2. Kaplan–Meier Curves for the Time to the First Transfusion during Treatment.

The P value for the comparison of times to the first transfusion between the two groups was calculated by the log-rank test.

Quality of Life

Assessments of the quality of life were performed with the use of two instruments, the FACIT-Fatigue instrument and the EORTC QLQ-C30 instrument. Patients in the eculizumab group had a mean increase (improvement) in scores on the FACIT-Fatigue instrument of 6.4±1.2 points from baseline to week 26, whereas in the placebo group the mean score decreased by 4.0±1.7 points during this period, for a total difference between the two groups of 10.4 points (Fig. 3). A mixed-model analysis of covariance was performed that showed a significant difference between the two groups ($P<0.001$).

With respect to the EORTC QLQ-C30 instrument, the eculizumab group had significant improvements in scores on the scale for global health status, on all five scales for functioning, on two of three symptom scales, and on three of six single-item measures, as compared with the placebo group ($P\leq0.01$ for each scale and measure) (Table 3).

SAFETY

No patients died during the study. Serious adverse events were reported in 13 patients: 4 in the eculizumab group and 9 in the placebo group (Table 4). No serious adverse events were considered to be treatment-related; all these patients recovered without sequelae. The most common adverse events reported in the eculizumab group were headache, nasopharyngitis, back pain, and nausea. Headache and back pain occurred more frequently in the eculizumab group than in the placebo group. The number of headaches that occurred was similar in the two groups after the first 2 weeks of therapy. There were no significant differences in the incidence rates between the two groups for any reported adverse event. A single thrombosis occurred in a patient in the placebo group.

One patient in each of the two groups had detectable levels of antibodies against eculizumab. The levels were low, were detected at a single visit, and in the patient receiving eculizumab, the antibodies did not affect complement inhibition.

DISCUSSION

Patients with PNH have chronic intravascular hemolysis with acute exacerbations. Anemia and the

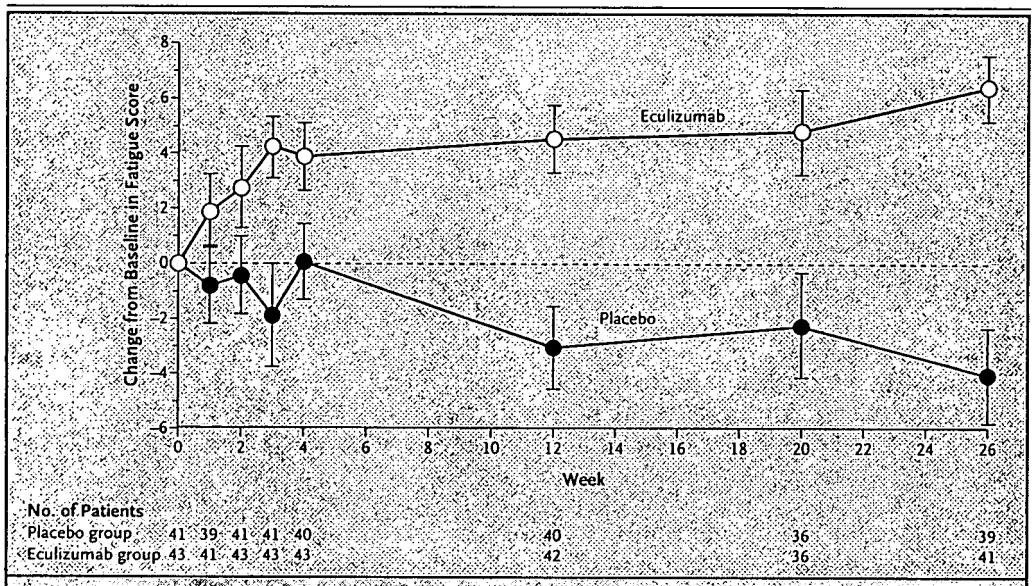

Figure 3. Change in Fatigue Scores from Baseline to Week 26.

Week 0 (baseline) represents the end of the observation period, within 10 days after the patient's receipt of the qualifying blood transfusion. Fatigue scores are therefore higher (indicating less fatigue) at week 0 than at the initial screening visits (data not shown). Values for the change from baseline (dashed line) represent means. A positive change from baseline indicates an improvement in fatigue and a negative change from baseline indicates a worsening in fatigue. I bars indicate the standard error.

need for transfusions to sustain hemoglobin levels occur frequently, as does deterioration of the patient's quality of life. In this study, in approximately half the patients treated with eculizumab, the end points of stabilization of hemoglobin levels and transfusion independence were reached, whereas none of the patients in the placebo group reached either of these end points. The median time to the first transfusion was 4 weeks in the placebo group and more than 6 months in the eculizumab group. The overall rate of transfusion was reduced by 73% in the eculizumab group. Even among patients receiving eculizumab in whom transfusion independence was not reached, the number of units of packed red cells transfused was reduced by 44%, as compared with patients in the placebo group (data not shown).

Intravascular hemolysis is central to the occurrence of serious coexisting conditions in patients with PNH and contributes to the risk of death among these patients.[9,12] Lactate dehydrogenase, a biochemical marker of hemolysis, was immediately reduced from approximately 10 times the upper limit of the normal range to normal levels or to just above normal levels in all patients in the eculizumab group. Residual low-level hemolysis in some patients despite terminal-complement blockade may be caused by an inherent decrease in the survival of PNH type III erythrocytes[19] or may be due to the fact that these cells are opsonized with C3b, which mediates extravascular clearance through the reticuloendothelial system.

Before treatment with eculizumab, the hemoglobin levels were maintained by transfusion. Therefore, the stabilization of hemoglobin levels with a concomitant cessation of or reduction in the number of transfusions indicates an increase in endogenous erythrocyte mass. The reduction in hemolysis with eculizumab results in a new steady-state hemoglobin level, as determined by a balance of the underlying bone marrow dysfunction, the increased half-life of PNH erythrocytes because of eculizumab therapy, and the new level of transfusions (if any) required.

For most patients with PNH, the quality of life is impaired, and the impairment has been attributed not only to anemia but also to excessive in-

Table 3. Change in the Quality of Life during Treatment.*

Scale	Mean Change in Score from Baseline to Week 26†		Absolute Difference	P Value‡
	Placebo Group	Eculizumab Group		
Global health status scale	−8.5	10.9	19.4	<0.001
Functioning scales				
Role	−6.9	17.9	24.8	<0.001
Social	2.0	16.7	14.7	0.003
Cognitive	−6.1	7.9	14.0	0.002
Physical	−3.5	9.4	12.9	<0.001
Emotional	−3.7	7.5	11.2	0.008
Symptom scales				
Fatigue	10.0	−16.9	26.9	<0.001
Pain	5.3	−12.3	17.6	0.002
Nausea and vomiting	2.8	−0.4	3.2	0.06
Single-item measures				
Dyspnea	8.9	−7.9	16.8	<0.001
Loss of appetite	3.3	−10.3	13.6	<0.001
Insomnia	4.9	−7.9	12.8	0.01
Financial difficulties	0.0	−10.3	10.3	0.19
Constipation	0.0	−6.3	6.3	0.20
Diarrhea	5.7	4.8	0.9	0.15

* The quality of life was assessed with the EORTC QLQ-C30 instrument.
† A positive value for a score on the scales for global health status and functioning indicates improvement, whereas a negative value for a score on the symptom scales and for a score on the single-item measures indicates improvement.
‡ P values are from a mixed model, with baseline scores as the covariate, treatment and time as fixed effects, and the patient identifier as a random effect.

travascular hemolysis and the scavenging of nitric oxide by cell-free hemoglobin.[9-11] In this study, the reduction in intravascular hemolysis with eculizumab, as compared with placebo, was associated with a significant improvement in fatigue, as assessed by scores on the FACIT-Fatigue instrument. Eculizumab increased the baseline score for fatigue by 6.4 points. A change of three or more points in scores on this instrument represents a clinically important difference.[20] Improvement with eculizumab in the fatigue component of the EORTC QLQ-C30 instrument provides additional evidence for the benefit shown by scores on the FACIT-Fatigue instrument. These improvements with eculizumab occurred without complete resolution of the anemia, providing further evidence of the contribution of hemolysis, in contrast to anemia, to the diminishing quality of life of patients with PNH. Clinical assessment of additional symptoms related to the quality of life of such patients, including abdominal pain, dysphagia, and erectile dysfunction, have also been reported to improve during eculizumab therapy.[21]

There were no deaths during the study, and only a single thrombotic event occurred in a patient in the placebo group. There were four serious adverse events in the eculizumab group and nine in the placebo group; all these patients recovered. The issue of possible protection against the risk of thrombosis through terminal complement inhibition with eculizumab is being evaluated in ongoing clinical studies of PNH. All 85 patients who completed the study elected to receive eculizumab in an open-label extension study.

The results of this randomized, double-blind, controlled study show that terminal complement inhibition with eculizumab reduces intravascular

Table 4. Adverse Events.*

Adverse Event	Placebo Group (N=44)	Eculizumab Group (N=43)
	no. (%)	
Total no. of serious adverse events	9 (20)	4 (9)
Exacerbation of PNH	3 (7)	1 (2)
Renal colic	0	1 (2)
Lumbar- or sacral-disk prolapse	0	1 (2)
α-Hemolytic streptococcal bacteremia	0	1 (2)
Central-line and urinary tract infections	1 (2)	0
Upper respiratory tract infection	1 (2)	0
Probable viral infection	1 (2)	0
Neutropenia	1 (2)	0
Cellulitis, folliculitis, and neutropenia	1 (2)	0
Anemia and pyrexia	1 (2)	0
Most frequent adverse events†		
Headache‡	12 (27)	19 (44)
Nasopharyngitis	8 (18)	10 (23)
Upper respiratory tract infection	10 (23)	6 (14)
Back pain	4 (9)	8 (19)
Nausea	5 (11)	7 (16)
Cough	4 (9)	5 (12)
Diarrhea	5 (11)	4 (9)
Arthralgia	5 (11)	3 (7)
Abdominal pain	5 (11)	2 (5)
Dizziness	5 (11)	2 (5)
Vomiting	5 (11)	2 (5)
Fatigue	1 (2)	5 (12)
Viral infection	5 (11)	1 (2)

* Adverse events were coded with the use of preferred terms from the MedDRA.
† The event occurred in at least 10% of patients in either group.
‡ After the first 2 weeks of treatment, 10 patients (23%) receiving placebo and 9 patients (21%) receiving eculizumab had headache.

hemolysis, reduces or eliminates the need for transfusion, and improves anemia, fatigue, and the quality of life in patients with PNH. The data provide support for the central role of intravascular hemolysis in the pathogenesis of the disease and indicate that eculizumab is an effective treatment in patients with PNH.

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APPENDIX

In addition to the authors, the following investigators and institutions participated in the TRIUMPH study: Australia — Princess Alexandra Hospital, Woolloongabba: A. Mills; Queen Elizabeth Hospital, Woodville South: J. Norman; Royal Melbourne Hospital, Parkville; Royal Perth Hospital, Perth, WA: R. Herrmann; Belgium — St. Luc University Hospital, UCL, Brussels: E. Van Den Neste; Canada — University of Alberta, Cross Cancer Institute, Edmonton, AB.: L. Larratt, A. Turner, M.A. Hamilton; Germany — Universitätsklinikum Essen, Essen: U. Dührsen; Medizinische Hochschule Hanover, Hanover: A. Ganser; Universitätsklinik Greifswald, Greifswald: M. Montemurro; Institut für Klinische Transfusionsmedizin and Immungenetik, University Hospital Ulm, Ulm; Saarland University Medical School, Hamburg; France — Hospital de l'Hotel-Dieu, Paris: B. Rio; Hospital St. Louis and INSERM, Paris; Ireland — St. James Hospital, Dublin; Italy — Ospedale San Martino, Genoa: A. Bacigalupo; Azienda-Ospedaliera Universitaria Careggi, Florence: E. Antonioli, G. Gianfaldoni, F. Mannelli, A. Bosi; Ospedale San Bortolo, Vicenza: F. Rodeghiero; Federico II University, Naples: B. Rotoli, F. Alfinito; Ospedale Maggiore di Milano, Milan: A. Zanella, C. Boschetti; Istituto Toscana Tumori, Florence; the Netherlands — Radboud University Medical Center, Nijmegen; Sweden — Lund University Hospital, Lund: P.-G. Nillson; Umea University Hospital, Umea: A. Wahlin; Stockholm South Hospital, Stockholm: J. Samuelsson, L.G. Lundberg, P. Andersson; United Kingdom — St. George's Hospital, London; Leeds General Infirmary, Leeds; Belfast City Hospital, Belfast: M.F. McMullin; United States — Washington University School of Medicine, St. Louis: M. Bessler, L. Andritsos, M. Blinder, S. Devine; Johns Hopkins University Medical Center, Baltimore; Memorial Sloan-Kettering Cancer Center, New York: H. Castro-Malaspina, D. Araten; Stanford University Medical Center, Stanford, CA: S. Coutre; Duke University Medical Center, Durham, NC: C. de Castro III; Cleveland Clinic Florida, Weston, FL: E. Stone; University of Pennsylvania, Philadelphia: B. Konkle; Massachusetts General Hospital, Boston: D. Kuter; Cleveland Clinic Foundation, Cleveland: A. Lichtin; New York University Clinical Cancer Center, New York: T. Moskovits, B.G. Raphael, E. Amorosi, K.B. Hymes, P. Cook; City of Hope National Medical Center, Duarte, CA; Indiana University Cancer Center, Indianapolis: R. Nelson; University of California at Los Angeles, Los Angeles: R. Paquette; Hartford Hospital, Hartford, CT: R. Siegel; National Heart, Lung, and Blood Institute, Bethesda, MD: B. Savani.

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INHIBITION OF COMPLEMENT ACTIVITY BY HUMANIZED ANTI-C5 ANTIBODY AND SINGLE-CHAIN Fv.

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Abstract—Activation of the complement system contributes significantly to the pathogenesis of numerous acute and chronic diseases. Recently, a monoclonal antibody (5G1.1) that recognizes the human complement protein C5, has been shown to effectively block C5 cleavage, thereby preventing the generation of the pro-inflammatory complement components C5a and C5b-9. Humanized 5G1.1 antibody, Fab and scFv molecules have been produced by grafting the complementarity determining regions of 5G1.1 on to human framework regions. Competitive ELISA analysis indicated that no framework changes were required in the humanized variable regions for retention of high affinity binding to C5, even at framework positions predicted by computer modeling to influence CDR canonical structure. The humanized Fab and scFv molecules blocked complement-mediated lysis of chicken erythrocytes and porcine aortic endothelial cells in a dose-dependent fashion, with complete complement inhibition occurring at a three-fold molar excess, relative to the human C5 concentration. In contrast to a previously characterized anti-C5 scFv molecule, the humanized h5G1.1 scFv also effectively blocked C5a generation. Finally, an intact humanized h5G1.1 antibody blocked human complement lytic activity at concentrations identical to the original murine monoclonal antibody. These results demonstrate that humanized h5G1.1 and its recombinant derivatives retain both the affinity and blocking functions of the murine 5G1.1 antibody, and suggest that these molecules may serve as potent inhibitors of complement-mediated pathology in human inflammatory diseases.

Key words: complement, C5, humanized antibody, Fab, scFv.

INTRODUCTION

Activated components of the complement system are believed to contribute to many of the pathophysiological mechanisms underlying numerous acute and chronic inflammatory processes (reviewed in Morgan (1994)). For example, cleavage of C3 generates both C3a, a relatively weak chemoattractant for neutrophils and C3b, a potent opsonin. The subsequent cleavage of C5 generates C5a, a potent chemo-attractant and activator of neutrophils, as well as C5b, which initiates deposition of the membrane attack complex C5b-9 on the cell surface. The recruitment of neutrophils contributes significantly to inflammatory tissue injury in numerous experimental systems (reviewed in Korthius and Granger (1994)), while C5b-9 deposition can result in cell activation due to calcium influx (Morgan, 1989), upregulation of cell surface adhesion molecules (Hattori *et al.*, 1989) or in cell lysis.

The complement cascade is regulated by endogenous inhibitors which act at numerous points including C1, C4, C3 convertase activity, C5 convertase activity and the formation of C5b-9 (Lachmann, 1991). Development of a soluble form of complement receptor type 1 (sCR1), an inhibitor of C3 convertase activity, has provided a means to examine the potential of complement inhibition for the amelioration of several disease processes including xenotransplant rejection (Pruitt *et al.*, 1994), lung and dermal immune complex-mediated injury (Mulligan *et al.*, 1992), experimental allergic encephalomyelitis (Piddlesden *et al.*, 1994), cardiopulmonary bypass (Moat *et al.*, 1992; Gillinov *et al.*, 1993) and reperfusion injury (Weisman *et al.*, 1990; Smith *et al.*, 1993; Pemberton *et al.*, 1993). However, inhibition of the complement cascade by sCR1 at the level of C3 convertase activity may be predicted to have significant clinical side effects. Generation of C3b is essential for the normal phagocytosis of bacterial and fungal pathogens as well as the clearance of circulating immune complexes (Liszewski and Atkinson, 1993). In fact, humans genetically deficient in C3 are subject to recurrent life-threatening infections and also suffer from a greatly increased incidence of autoimmune diseases such as systemic lupus erythrematosus and glomerulonephritis (Ross and Densen, 1984).

To avoid these complications, we have focused on the

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Exhibit H

inhibition of the complement cascade at the level of C5, thereby blocking formation of the proinflammatory complement activation products C5a and C5b-9, while preserving the generation of C3b. Patients with a genetic deficiency of C5 suffer only from an increased incidence of Neisserial infections, which are in fact milder than infections in patients having normal levels of C5 (Ross and Densen, 1984). As there are no endogenous inhibitors specific for the C5 convertases, monoclonal antibodies were developed, which bind human C5 and prevent its cleavage by C5 convertase (Würzner *et al.*, 1991; Khroshus *et al.*, 1995). These antibodies inhibit complement-mediated acute cardiac tissue injury occurring in perfused heart models of xenotransplantation (Kroshus *et al.*, 1995) and block leukocyte and platelet activation in an *ex vivo* recirculation model of cardiopulmonary bypass (Rinder *et al.*, 1995). Additionally, in pigs, a monoclonal antibody against C5a reduces myocardial infarct size by 35% (Amsterdam *et al.*, 1995). Finally, a monoclonal antibody against mouse C5 (Frei *et al.*, 1987) blocks progression of established arthritis in a murine collagen-induced arthritis model (Wang *et al.*, 1995) and prevents development of glomerulonephritis in a murine model of systemic lupus erythromatosis (Wang *et al.*, 1995).

The desired pharmacological properties of complement inhibitors are dramatically different for acute versus chronic settings. In acute settings, a complement inhibitor should have a relatively short serum half-life, coupled with a rapid rate of tissue penetration, since complement activation occurs within the tissue (Schäfer *et al.*, 1986; Hugo *et al.*, 1990). Large molecules such as monoclonal antibodies have very slow rates of tissue penetration as compared to smaller molecules such as Fab fragments (Covell *et al.*, 1986) or single chain Fv (scFv) molecules (Yokota *et al.*, 1992). A complement inhibitor used in chronic settings should have a prolonged serum half-life and not provoke an immune response. In humans, murine monoclonal antibodies have a half-life of approximately 1 day (LoBuglio *et al.*, 1993) and uniformly provoke an immune response (Khazaeli *et al.*, 1994). These problems have been addressed by the development of humanized monoclonal antibodies which have serum half-lives of several days (LoBuglio *et al.*, 1989; Khazaeli *et al.*, 1991; Saleh *et al.*, 1992; Schüpbach *et al.*, 1993) as well as greatly diminished immunogenicity (LoBuglio *et al.*, 1993)

In this study, variants of the 5G1.1 monoclonal antibody have been engineered, which can be used in either acute or chronic settings. Firstly, the 5G1.1 variable regions were humanized using the CDR-grafting technique (Riechmann *et al.*, 1988). The humanized variable regions were then used to construct humanized antibody, Fab and scFv molecules which all maintained a high affinity for human C5 and blocked the generation of both C5a and C5b-9.

MATERIALS AND METHODS

Cloning of 5G1.1 variable region genes

For N-terminal amino acid sequencing, 75 μg 5G1.1 mAb was subjected to SDS–PAGE under reducing conditions and transferred to ProBlott membrane (Applied Biosystems, Foster City, CA, U.S.A.) as previously described (Evans *et al.*, 1995). Protein bands were localized by staining with Ponceau S, excised and subjected to amino acid sequence analysis using Edman chemistry, performed on a pulsed liquid protein sequencer (ABI model 477A) with the PTH amino acids analysed using an on-line microbore HPLC system (ABI model 120A).

To deblock the amino terminus of the 5G1.1 heavy chain, 10 mg 5G1.1 monoclonal antibody was exchanged into reducing buffer (5 M guanidine–HCl, 50 mM Tris–HCl, 10 mM dithiothreitol, pH 8.5), using a PD-10 column (Pharmacia, Piscataway, NJ, U.S.A.). After incubation for 1 hr at room temperature, 50 mM iodoacetamide was added and the incubation allowed to continue for 30 min. The carbamidomethylated light and heavy chains were then separated by size exclusion chromatography on a Superose 12 (Pharmacia) column equilibrated with 5 M guanidine–HCl, 50 mM Tris–HCl (pH 8.5). The purified light chain was exchanged into 50 mM sodium phosphate (pH 7.0), using a PD-10 column, digested using 0.5 mU pyroglutamate aminopeptidase (PanVera, Madison, WI, U.S.A.) per nmol of heavy chain protein and sequenced as previously described. For determination of internal amino acid sequence, the isolated light chain was exchanged into 2 M urea, 25 mM Tris–HCl, 1 mM EDTA (pH 8.0) and incubated at 37°C overnight with endoproteinase Lys-C (Promega, Madison, WI, U.S.A.) at a protease:protein ratio of 1:40. The digested material was run on a C18 reversed phase HPLC column (Beckman Instruments, Fullerton, CA, U.S.A.) and eluted using a linear acetonitrile gradient (0–50%) in 0.1% trifluoroacetic acid. Peaks were subjected to amino acid sequence analysis as previously described.

Cloning of the 5G1.1 heavy chain variable region (VH) and light chain variable region (VL) was initially performed using a set of commercially available primers (Mouse Ig-Primer Set, catalogue number 69831-1; Novagen, Madison, WI, U.S.A.) essentially as previously described (Evans *et al.*, 1995). Comparison to the obtained peptide sequences confirmed that the correct VH had been cloned, whereas neither of the two light chain amino acid sequences were present in the cloned VL. To isolate the 5G1.1 VL, the UWGCG program TFASTA was used to search the GenBank rodent subdirectory with the amino acid sequence obtained from the purified light chain (IQMTQSPASLSASVGETVT). An exact match to this sequence was located in the murine germline gene encoding the v-kappa k2 variable region (Seidman *et al.*, 1978). The DNA sequence of this germline gene was used to design an oligonucleotide for use as a variable region 5′-primer for isolation of the correct VL gene.

Humanization of 5G1.1 variable regions

Two humanized variants of the 5G1.1 VL and VH regions were constructed by CDR grafting (Reichmann *et al.*, 1988). For CDR grafting, the 5G1.1 heavy and light complementarity determining regions were intro-

duced into the human heavy variable region H20C3H (Weng *et al.*, 1992) to yield h5G1.1VHC (Fig. 1A) or the human light variable region L23 (Klein *et al.*, 1993) to yield h5G1.1VLC (Fig. 1B), respectively. Construction of each CDR-grafted variable region was performed by PCR amplification, using two synthetic overlapping oligonucleotides of approximately 200 nucleotides in length. The cloned PCR products were sequenced on both strands in their entirety to ensure the absence of PCR-introduced errors. Murine amino acids were introduced into the human framework regions at position 71 in the light variable region and positions 71 and 78 in the heavy variable region by PCR mutagenesis.

Expression and purification of Fab

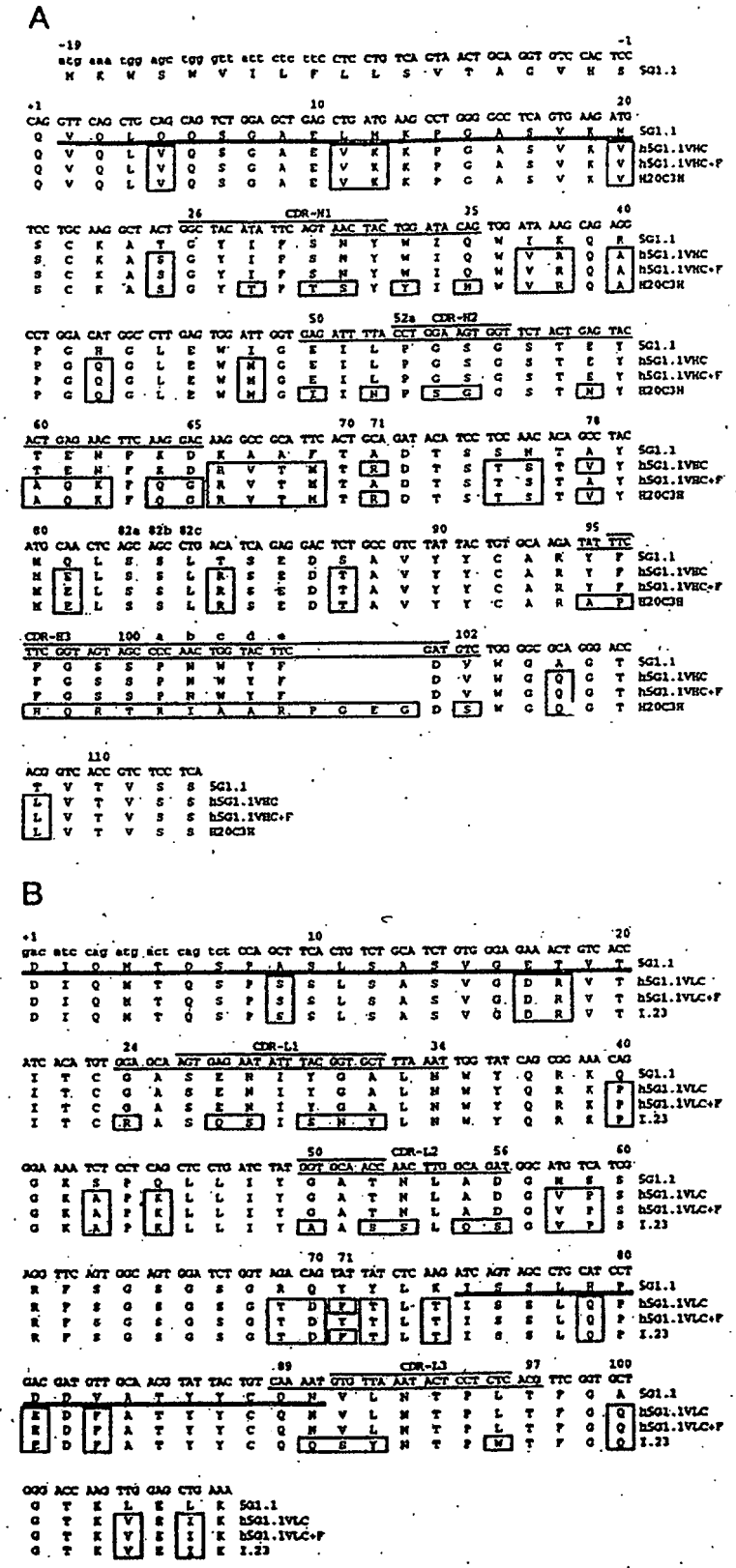
Expression of chimeric and humanized 5G1.1 Fab molecules was achieved using the mammalian 293-EBNA expression system essentially as previously described (Evans *et al.*, 1995*b*). Briefly, a plasmid capable of producing a chimeric Fab was constructed by fusion of the murine VL to the human kappa (*Inv*3 allele) constant region and fusion of the murine VH to the human IgG1 (*G1m*(17) allele) Fd in the expression plasmid pAPEX-3P. Since the cloning strategy for the 5G1.1 VL did not obtain the leader sequence, the leader sequence of the human CD59 protein was cloned in frame to allow secretion of the chimeric light chain cDNA. Similarly, a plasmid capable of expressing the CDR grafted Fab, h5G1.1 Fab (CDR), was constructed by cloning the variable regions h5G1.1VHC and h5G1.1VLC into pAPEX-3P. Finally, a plasmid capable of expressing the CDR grafted Fab containing framework changes, h5G1.1 Fab (CDR + FW), was constructed by cloning the variable regions h5G1.1VHC+F and h5G1.1VLC+F into pAPEX-3P. 293-EBNA cells (Invitrogen, San Diego, CA, U.S.A.) were transfected with the pAPEX-3P expression plasmids and selected using 1 μg/ml puromycin. Secreted Fabs were purified using protein G Sepharose chromatography as described (Evans *et al.*, 1995*b*), dialysed into PBS, and stored at 4°C. Protein concentrations were determined by the Bradford assay (Bio-Rad, Melville, NY, U.S.A.).

Construction of a humanized scFv

Two scFv proteins were constructed by the overlapping PCR technique. The h5G1.1 scFv (CDR) contained the h5G1.1VLC variable region, linked to the h5G1.1VHC variable region; while the h5G1.1 scFv (CDR + FW) contained the h5G1.1VLC+F variable region, linked to the h5G1.1VHC+F variable region. These scFv cDNAs were cloned into a plasmid in which expression is driven by the trc promoter. *Escerichia coli* strain ME1 was transformed with the resulting expression plasmids. Transformants were grown at 37°C in 2 l Applikon glass vessel fermentors containing Terrific Broth (1.2% (w/v) bactotryptone, 2.4% (w/v) bacto-yeast extract, 0.4% (v/v) glycerol, 90 mM potassium phosphate, pH 7.0), supplemented with 100 μg/ml ampicillin. The production of recombinant scFv was induced by the addition of 1 mM isopropylthio-β-D-galactoside when the OD_{550} of the culture reached 10. After an additional 3 hr incubation at 37°C, the cells were harvested by centrifugation and the cell pellets stored at −20 C. Cells were resuspended in 1 mM EDTA (pH 5.0), at 10 ml g weight and lysed by a single pass through a microfluidizer M110-T (Microfluidics Corp., Newton, MA, U.S.A.). After centrifugation at 17 500g for 15 min, the resulting inclusion body pellet was resuspended in 20 mM Tris–HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl, 0.15% (w/v) deoxycholate at 10 ml/g inclusion body, using a Tekmar polytron. The inclusion bodies were again pelleted by centrifugation at 17 500*g* for 15 min and resuspended in 20 mM Tris–HCl (pH 9.0) and 8 M urea at 10 ml/g. After stirring for 1 hr, the sample was centrifuged at 14 000*g* for 30 min to pellet remaining insoluble material. The resulting supernatant was diluted 10-fold with 20 mM Tris–HCl (pH 9.0), 7 M urea and 50 μM cupric sulfate, and allowed to stir for at least 16 hr at 4°C to refold the scFv. Biocryl BPA-1000 (TosoHaas, Montgomeryville, PA, U.S.A.) was then added as a flocculation agent at 3 μl/ml (or at 10 μl/ml for the anti-mouse C5 scFv). After stirring for 5 min, the sample was centrifuged at 15 000*g* for 10 min to pellet insoluble material. The supernatant was exchanged into 20 mM Tris (pH 9.0), 1 mM EDTA by diafiltration and concentrated by ultrafiltration using a stirred cell, fitted with a YM10 membrane (Amicon, Beverly, MA, U.S.A.). Properly refolded scFv was separated from aggregated material and contaminating proteins by anion exchange chromatography using Q Sepharose Fast Flow (Pharmacia). Bound scFv was eluted with 20 mM Tris–HCL (pH 9.0) and 1 mM EDTA containing a linear NaCl gradient (0–0.5 M). The fractions containing the scFv were combined, concentrated by ultrafiltration using a stirred cell fitted with a YM10 membrane and applied to a Sephacryl S200 HR 26/100 column (Pharmacia) equilibrated in 20 mM Tris–HCl, pH 9.0, 1 mM EDTA and 150 mM NaCl. Fractions containing the scFv were combined, exchanged into PBS by diafiltration, concentrated by ultrafiltration, filtered through a 0.22 mm Millex-GV filter (Millipore, Bedford, MA, U.S.A.) and stored at 4°C. Protein concentration was determined by the Bradford assay.

ELISA binding assays

For detection of recombinant Fab binding to human C5, F96 PolySorp microtiter plates (Nunc, Naperville, IL, U.S.A.) were coated overnight at 4°C with 0.1 mg/well human C5 (Quidel, San Diego, CA, U.S.A.) at a concentration of 2 mg/ml in 0.1 M Na_2CO_3, pH 9.6. The plates were then washed three times with 100 μl/well wash buffer (PBS containing with 0.5% (v/v) Tween 20) and blocked with 100 μl/well blocking buffer (PBS supplemented with 1% (w/v) bovine serum albumin, fraction V and 0.5% (v/v) Tween 20) at 37°C for 1 hr. The plates were again washed three times with wash buffer and incubated with 50 μl/well blocking buffer, containing 30 ng/ml murine 5G1.1 mAb, plus the indicated concentrations of inhibitor, at 37°C for 2 hr. The plates were


A
B
CDR-H1
CDR-H2
CDR-H3
CDR-L1
CDR-L2
CDR-L3
hSG1.1VHC
hSG1.1VHC+F
H20C3H
hSG1.1VLC
hSG1.1VLC+F
I.23

again washed three times with wash buffer, and incubated with 50 μl/well blocking buffer, containing peroxidase-conjugated goat anti-mouse IgG Fc antibody, at a 2000-fold dilution (Sigma, St. Louis, MO, U.S.A.), at 37°C for 1 hr. After three final washes, the plate was developed with 50 μl/well substrate buffer (0.05 M phosphate-citrate buffer, pH 5.0, containing 0.3 mg/ml sodium perborate and 0.4 mg/ml *o*-phenylenediamine dihydrochloride). Reactions were stopped by the addition of 50 μl/well 1 M sulfuric acid and quantified using a Bio-Rad model 3550 plate reader set at 490 nm.

Hemolytic and C5a generation assays

The antibody, Fab, or scFv was pre-incubated with the indicated percent (v/v) human serum in 0.1 ml veronal buffered saline containing 1% gelatin ($GVBS^{++}$) for 30 min at room temperature. Chicken erythrocytes (Lampire Biological, Pipersville, PA, U.S.A.) were washed four times with $GVBS^{++}$ and resuspended at 5×10^7 cells/ml in $GVBS^{++}$, containing 1 μg/ml anti-chicken erythrocyte IgG (Inter-cell Technologies, Hopewell, NJ, U.S.A.). After incubation at 4°C for 15 min, the cells were washed twice with $GVBS^{++}$ and 3×10^6 cells were added to the pre-incubated human serum. After further incubation for 30 min at 37°C, the cells were pelleted by centrifugation at 10 000*g* for 2 min and the supernatant was assayed for released hemoglobin by measurement of the absorbance at 415 nm, and for C5a content by sandwich ELISA as described (Würzner *et al.*, 1991).

Porcine aortic endothelial cell (PAEC) lysis assays

PAEC were obtained from Cell Systems (Kirkland, WA, U.S.A.) at passage 1 and maintained in M199 medium, supplemented with 20% fetal calf serum, 20 mM HEPES, 10 μg/ml heparin (Sigma) and 5 μg/ml ECGF (Biomedical Technologies, Stoughton, MA, U.S.A.). PAECs at passage 3 were plated at 10 000 cells/well in a 96-well Falcon flat-bottomed microtiter plate. The following day, cells were washed twice with Hank's balanced salt solution, containing 1% (w/v) bovine serum albumin (HBSS/BSA) and incubated with 10 μM calcein AM (Molecular Probes, Eugene, OR, U.S.A.) in HBSS/BSA for 30 min at 37°C. Wells were washed twice with HBSS/BSA and incubated with 0.85 mg/ml rabbit anti-PAEC antibody (Kennedy *et al.*, 1994) for 30 min at 37°C. Wells were again washed twice with HBSS/BSA and incubated with HBSS containing 20% (v/v) human serum plus various concentrations of inhibitors for 30 min at 37 C. The supernatant was then transferred to a new 96-well Falcon flat-bottom microtiter plate and the remaining cells were solubilized with 1% SDS. The fluorescence of the supernatants and solubilized cells was determined using a Cytofluor 2350 (Millipore) with an excitation wavelength of 485 nm and an emmision wavelength of 530 nm. Percentage dye release was defined as the ratio of supernatant fluorescence to total (supernatant plus cell) fluorescence. Supernatant fluorescence in the absence of human serum was subtracted as background release.

RESULTS

Humanization of 5G1.1

The variable regions of the anti-C5 monoclonal antibody 5G1.1 were cloned by PCR amplification using 5′ primers, based on amino acid sequencing (for the light variable region) or degenerate 5′ primers (for the heavy variable region), coupled with 3′ primers specific for murine constant region sequences. Amino acid sequence analysis confirmed that the appropriate heavy and light variable regions had been cloned (Fig. 1). The 5G1.1 heavy variable region was most similar to V_H genes belonging to the V_H1/J558 family (Kofler *et al.*, 1992) and utilized the J_H1 gene. The most similar cloned murine V_H region was from the IgM secreting hybridoma 19.1.2 (Akolkar *et al.*, 1987). The 5G1.1 V_H differed from this clone at positions 20, 37 and 68 in framework regions, as well as at positions 28, 31 and 35 in CDR-H1, positions 58 and 65 in CDR-H2 and positions 95, 96 and 97 in CDR-H3. The 5G1.1 light variable region was most similar to the NYC V_κ gene (Jack *et al.*, 1992) and utilized the mouse $J_\kappa5$ gene (Hieter *et al.*, 1980), with amino acid differences present at framework position 72 in framework region 3 and at position 93 in CDR-L3.

For humanization, the 5G1.1 heavy variable regions CDRs were transposed into the human variable region

Fig. 1. Sequence of the 5G1.1 heavy (A) and light (B) variable regions. The DNA sequence and the translated amino acid sequence of the cloned 5G1.1 variable regions are shown. Amino acid position is numbered according to Kabat *et al.* (1992), with the complementarity determining regions according to the hypervariable sequence definition (Kabat *et al.*, 1992) or the structural variability definition (Chothia and Lesk, 1987) underlined and overlined, respectively. Lower case letters indicate nucleotide sequences derived from primers used for cloning. Amino acid sequences obtained from protein sequencing are indicated by double underlines. The protein sequences of the human variable regions H20C3H and I.23 are shown below the appropriate 5G1.1 variable regions. h5G1.1VHC and h5G1.1VLC denote humanized heavy and light variable regions constructed by grafting the CDRs from 5G1.1 on to the H20C3H and I.23 human framework regions. The variable regions h5G1.1 VHC+F and h5G1.1VLC+F contain murine amino acids at framework positions 71 and 78 in the heavy variable region and position 71 in the light variable region in addition to the murine CDR sequences. Amino acids in the human variable regions and the humanized 5G1.1 variable regions which differ from the murine 5G1.1 sequences are boxed.

H20C3H (Weng *et al.*, 1992). This human V_H was derived from the human genomic V_H gene HG3 (Rechavi *et al.*, 1983), belonging to the V_H1 family and the human genomic J_H5 gene, and contains no changes in the framework regions from these genomic genes. The 5G1.1 light variable region CDRs were grafted into the human light variable region L23 (Klein *et al.*, 1993). This human V_L region was derived from the human $V_\kappa1$ family gene O12 (Klein *et al.*, 1993) and the genomic $J_\kappa1$ gene, with the introduction of an Arg residue in framework region 2 at position 38 in the L23 cDNA as compared to the Gln residue encoded in the O12 genomic gene. Initial humanized 5G1.1 variable heavy and light regions were constructed by introducing the 5G1.1 CDRs into these human frameworks and are designated as h5G1.1 VHC and 5G1.1 VLC (Fig. 1).

Computer modeling of antibody variable regions has indicated that amino acid residues within the framework regions of a variable region can influence the structure of the CDRs. Comparison of the murine 5G1.1 variable region with the human acceptor variable regions H20C3H and L23 suggested that the murine and human framework regions differed at three positions important for CDR structure. In the light chain, the presence of either Phe or Tyr at framework position 71 determines the canonical structure of CDR-L1 (Chothia and Lesk, 1987; Foote and Winter, 1992). Since L23 contains a Phe residueat position 71, whereas 5G1.1 contains a Tyr residue, the Phe residue in h5G1.1 VLC was changed to a Tyr residue to create the variable region h5G1.1 VLC+F (Fig. 1). Similarly, the human H20C3H and murine 5G1.1 V_H regions differ at framework positions 71 and 78, which have been suggested to be important for maintaining the function of humanized antibodies (Carter *et al.*, 1992; Chothia *et al.*, 1989; Tramontano *et al.*, 1990; Foote and Winter, 1992). The murine amino acids were, therefore, introduced at these positions to create the variable region h5G1.1 VHC+F (Fig. 1).

Since choice of human antibody isotype can influence antibody avidity whereas isotype choice does not influence Fab affinity (Morelock *et al.*, 1994), initial analysis of the humanization procedure was performed using humanized Fab fragments. The murine 5G1.1 V_L and its humanized variants h5G1.1VLC and h5G1.1VLC+F were fused to the human kappa constant region (Inv3 allele), to create an intact light chain. The 5G1.1 V_H and its humanized variants h5G1.1VHC and h5G1.1VHC+F were fused to constant region 1 of human IgG1 (*G1m*(17) allele) to create an intact Fd. Three humanized Fabs were produced in 293-EBNA cells

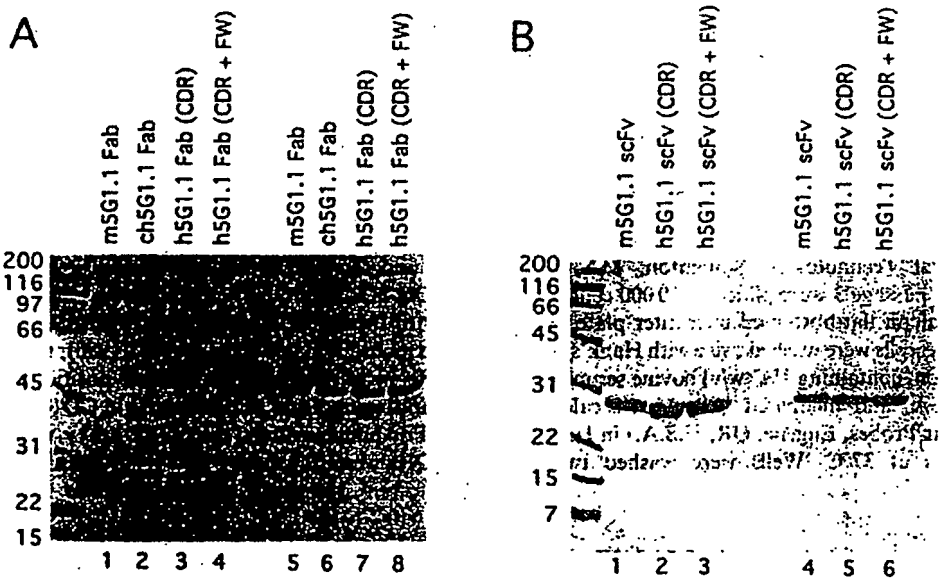


Fig. 2. SDS–PAGE analysis of purified recombinant proteins. (A) Fab fragments from the murine 5G1.1 mAb (m5G1.1 Fab) were produced by papain digestion of the m5G1.1 mAb followed by protein A chromatography to remove undigested antibody and Fc fragments. Recombinant chimeric Fab (ch5G1.1 Fab), CDR-grafted Fab (h5G1.1 Fab (CDR)), or CDR-grafted Fab containing murine framework amino acids (h5G1.1 Fab (CDR + FW)) were produced in 293 cells and purified from conditioned, serum-free medium using protein G-sepharose chromatography. Protein samples (10 mg) were subjected to SDS–PAGE under reducing (lanes 1–4) or non-reducing conditions (5–8) and stained with Coomassie R-250. (B) scFv molecules containing the murine variable regions (m5G1.1 scFv), the CDR-grafted variable regions (h5G1.1 scFv (CDR)), or the CDR-grafted variable regions containing murine framework amino acids (h5G1.1 scFv (CDR + FW)) were produced in *E. coli* and purified, as described in Materials and Methods. Ten micrograms of each purified scFv was subjected to SDS–PAGE under reducing (lanes 1–3) or non-reducing (lanes 4–6) conditions and stained with Coomassie R-250.

by co-expression of light chain and Fd: (1) ch5G1.1 Fab, a recombinant chimeric Fab containing the murine 5G1.1 variable regions; (2) h5G1.1 Fab (CDR), a recombinant humanized Fab containing the h5G1.1VHC and h5G1.1VLC variable regions; and (3) h5G1.1 Fab (CDR + FW), a recombinant humanized Fab containing the h5G1.1VHC+F and h5G1.1VLC+F variable regions. SDS-PAGE analysis of the purified recombinant Fabs revealed a single band, which comigrated with Fab produced from 5G1.1 under non-reducing conditions and resolved into stoichiometric amounts of Fd and light chain bands, under reducing conditions (Fig. 2).

Binding of recombinant Fab and scFv to human C5

The affinities of Fab produced by papain digestion of the 5G1.1 mAb and the recombinant humanized Fabs were initially compared in a competitive binding ELISA, in which increasing concentrations of test Fab were used to displace murine 5G1.1 mAb binding to human C5 (Fig. 3). Biochemically produced Fab (m5G1.1 Fab) inhibited binding with an IC_{50} of approximately 2 nM. Similarly, the recombinant ch5G1.1 Fab, h5G1.1 Fab (CDR) and h5G1.1 (CDR+ FW) all inhibited binding by 50% at approximately 2 nM.

Although the humanized Fabs retained biological activity, neither the h5G1.1 Fab (CDR) nor the h5G1.1 Fab (CDR + FW) could be produced in significant quantities in *E. coli* (data not shown). Three scFv molecules were, therefore, constructed (Fig. 4); (1) m5G1.1 scFv, containing the murine variable light region and murine variable heavy region joined by a linker; (2) h5G1.1 scFv (CDR), containing the h5G1.1VHC and h5G1.1VLC variable regions; and (3) h5G1.1 scFv (CDR + FW), containing the h5G1.1VHC+F and h5G1.1VLC+F variable regions. These scFv molecules were produced as inclusion bodies in *E. coli*, refolded, and purified by anion exchange and sizing chromatography (Fig. 2). Gel filtration chromatography coupled with light scattering analysis demonstrated that at a concentration of 2.0 mg/ml (75 μM) the purified h5G1.1 scFv (CDR) was predominantly in the form of dimers. However, dilution of the h5G1.1 scFv resulted in a rapid shift of the equilibrium towards the monomer state (Thomas *et al.*, 1996). In the competition ELISA analysis, the m5G1.1 scFv inhibited binding of 5G1.1 mAb to human C5 with an IC_{50} value of 2 nM (Fig. 5), identical to the value obtained for the chimeric Fab (Fig. 3). Similarly, both the h5G1.1 scFv (CDR) and h5G1.1 scFv (CDR + FW) inhibited 5G1.1 mAb binding with IC_{50} values of 1.5 and 2 nM, respectively. In contrast, an scFv specific for mouse C5 (anti-mC5, Fig. 5) did not inhibit 5G1.1 binding, even at the highest concentrations assayed. Together these results indicated that the refolded scFv molecules bound to human C5 with an affinity equivalent to the Fab molecules produced in mammalian cells. Furthermore, no effect of the framework alterations could be discerned, based on the binding affinity of either the Fab or scFv molecules.

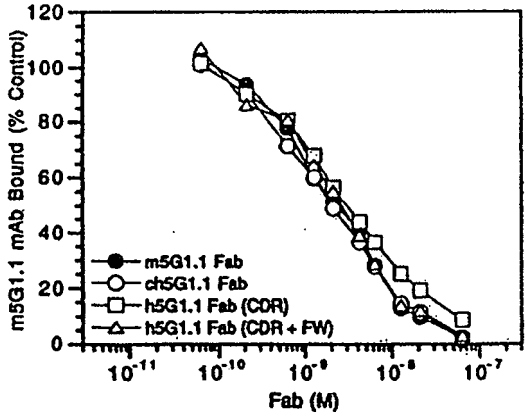


Fig. 3. Inhibition of 5G1.1 mAb binding to human C5 by recombinant Fab. 5G1.1 (0.2 nM) mAb plus the indicated concentrations of recombinant Fab were incubated together, for 2 hr at 37°C in microtiter plates coated with human C5. After washing, bound 5G1.1 mAb was detected using peroxidase-conjugated goat anti-mouse IgG (Fc specific) with quantitation performed by monitoring hydrolysis of *o*-phenylenediamine dihydrochloride at 490 nm. One hundred percent binding was defined in each experiment as the signal produced by binding of 5G1.1 mAb in the absence of competitor Fab. Data shown are mean values obtained from four to eight independent assays, with each datum point assayed in duplicate in each experiment.

Inhibition of C5b-9 formation by humanized Fab and scFv

The ability of the humanized Fab and scFv proteins to inhibit the formation of the C5b-9 membrane attack complex was evaluated using a hemolytic assay, in which the deposition of membrane attack complex on antibody-sensitized chicken erythrocytes is assessed by measuring hemoglobin release. Hemolysis was inhibited by greater than 90% when 0.25 μM of biochemically prepared murine 5G1.1 Fab, or each of the recombinant Fab molecules were added to the assay (Fig. 6A). This concentration corresponds to an approximate three-fold molar excess of Fab, as compared to the predicted human C5 concentration (0.08 μM) present in the assay. As with the Fab molecules, the scFv molecules also inhibited chicken erythrocyte hemolysis when present at a three-fold molar excess (Fig. 6B). The inhibition curves obtained with the scFv molecules were identical to those obtained with the Fab (Fig. 6B). The ability of the scFv to protect mammalian cells from complement-mediated damage was assessed by measuring their ability to block the lysis of antibody-coated porcine aortic endothelial cells (PAEC) by 20% human serum. The m5G1.1 scFv, h5G1.1 scFv (CDR) and h5G1.1 scFv (CDR + FW) all inhibited lysis of the PAEC when present at a three-fold molar excess, while the scFv specific for mouse C5 did not inhibit lysis, even at the highest concentration tested (Fig. 7A). The specificity of the 5G1.1 mAb for human C5 was maintained by the scFv, as neither the 5G1.1

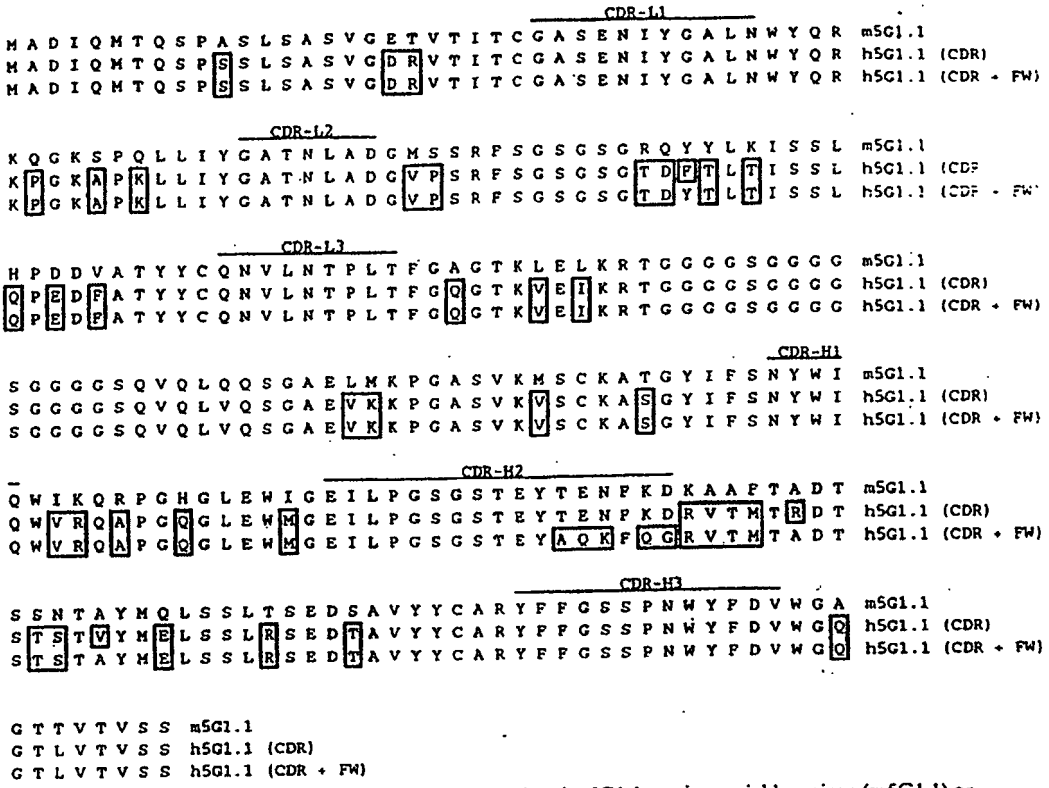


Fig. 4. Sequence of scFv molecules constructed using the 5G1.1 murine variable regions (m5G1.1) or humanized variable regions. The h5G1.1 (CDR) scFv contains the variable regions h5G1.1 VLC and h5G1.1 VHC, while the h5G1.1 scFv (CDR + FW) contains the variable regions h5G1.1 VLC+F and h5G1.1 VHC+F. The positions of the CDRs, as defined by Kabat *et al.* (1992), are indicated. Amino acid sequence analysis indicated removal of the initiator methionine, with the alanine residue at position two being the amino terminal residue of the bacterially produced scFv molecules. Amino acids in the humanized 5G1.1 variable regions which differ from the murine 5G1.1 sequences are boxed.

scFv, the h5G1.1 scFv (CDR) nor the h5G1.1 scFv (CDR + FW) could inhibit the lysis of PAEC by rat serum (Fig. 7B).

A critical parameter for use of an scFv as a C5 inhibitor is retention of the ability to block C5a generation. Previously, it has been found that a chimeric Fab, constructed from the monoclonal antibody N19-8 retained the ability to block both the formation of C5b-9 and generation of C5a, whereas an scFv constructed using the N19-8 variable regions retained the ability to block the formation of C5b-9, but only weakly inhibited the generation of C5a (Evans *et al.*, 1995). In contrast, analysis of supernatants obtained from the chicken erythrocyte hemolytic assays for C5a content (Fig. 8) indicated that equivalent concentrations of the humanized 5G1.1 Fab or scFv molecules blocked the generation of C5a. The concentration of inhibitor required for inhibition of C5a generation (Fig. 8) was comparable to the concentration of inhibitor required for inhibition of lysis (Fig. 6B).

Constuction of a humanized h5G1.1 antibody

Having demonstrated the effective humanization of the 5G1.1 variable regions, an intact humanized antibody (IgG4 isotype) was constructed and produced in 293-EBNA cells. The avidity of this humanized antibody (h5G1.1 HuG4) for human C5, was compared to the murine 5G1.1 mAb by determining the ability of each to compete binding of biotinylated 5G1.1 mAb to C5 (Fig. 9). The humanized h5G1.1 mAb had a two-fold lower avidity than the murine antibody. However, the humanized h5G1.1 HuG4 antibody was equipotent with the murine antibody at protecting PAEC from lysis by human serum, with a 0.5-fold molar ratio of antibody to C5 (1:1 ratio of antibody binding sites to C5) completely inhibiting lysis of the PAEC (Fig. 10).

DISCUSSION

This article describes the successful humanization of the anti-human C5 specific monoclonal antibody 5G1.1, utilizing the CDR-grafting technique. Although the original description of the CDR-grafting technique described the maintenance of antibody specificity by the transfer of the CDRs alone (Riechmann *et al.*; 1988), in most cases the transfer of a number of murine framework amino acids in addition to the CDRs has been found to be

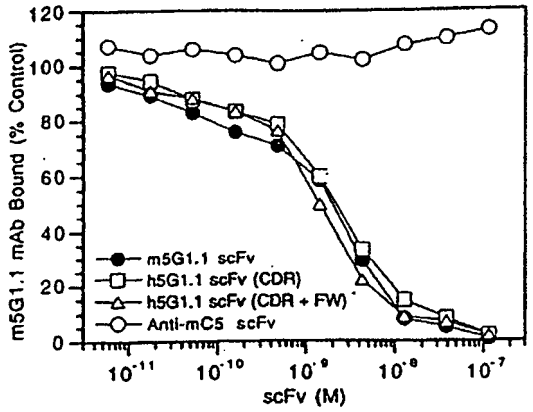


Fig. 5. Inhibition of 5G1.1 mAb binding to human C5 by recombinant scFv. 5G1.1 mAb (0.2 nM) plus the indicated concentrations of recombinant scFv were incubated together for 2 hr at 37°C, in microtiter plates coated directly with human C5. After washing, bound 5G1.1 mAb was detected using peroxidase-conjugated goat anti-mouse IgG (Fc specific) with quantitation performed by monitoring hydrolysis of *o*-phenylenediamine dihydrochloride at 490 nm. One hundred percent binding was defined in each experiment as the signal produced by binding of 5G1.1 mAb in the absence of competitor scFv. Data shown are mean values obtained from four to 10 independent assays, with each datum point assayed in duplicate in each experiment.

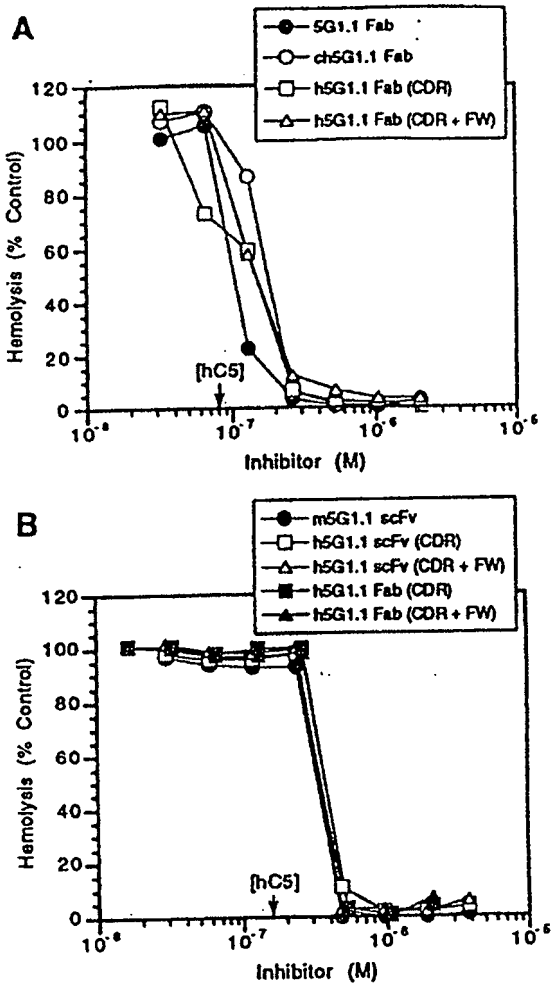


Fig. 6. Inhibition of chicken erythrocyte lysis by recombinant Fab and scFv. Twenty percent (A), or 40% (B), human serum in GVBS^{++} was pre-incubated for 30 min at room temperature, with the indicated concentrations of competitors. Chicken erythrocytes, precoated with rabbit anti-chicken erythrocyte antibody, were added and the incubation was continued for 30 min at 37°C. The erythrocytes were then pelleted and the supernatants assayed for released hemoglobin by spectrophotometry (A_{415}). The A_{415} absorbance value obtained in the absence of inhibitor was defined as 100% hemolysis. Data shown are the average results of two to four independent determinations. The predicted hC5 concentrations in 20% and 40% serum are indicated by arrows.

necessary for retention of high affinity binding to the antigen (Winter and Milstein, 1991). These framework amino acids are believed to influence the structure of the CDRs. In particular, heavy variable region framework amino acid 71 determines the canonical structure of CDR-H2 (Tramontano *et al.*, 1990), while light variable region framework amino acid 71 determines the canonical structure of CDR-L1 (Chothia and Lesk, 1987; Foote and Winter, 1992). The human variable regions chosen for grafting of the 5G1.1 CDRs differed from the murine variable regions at heavy region framework position 71 and light region framework position 71. Therefore, humanized variable regions were constructed and designated h5G1.1 (CDR + FW), which maintained the murine amino acids at both of these positions.

The importance of these framework amino acids on the humanization process was initially assessed using recombinant Fab produced in 293 cells. Both the Fab containing the framework changes, h5G1.1 Fab (CDR + FW) and the Fab with unmodified human framework regions, h5G1.1 Fab (CDR), had the same binding affinity as murine Fab, when assayed by competition ELISA (Fig. 3). Similarly, no difference could be discerned between h5G1.1 Fab (CDR), h5G1.1 Fab (CDR + FW) and the murine Fab in their ability to inhibit human complement-mediated lysis of chicken erythrocytes (Fig. 6A). The h5G1.1 Fab (CDR) and h5G1.1 Fab (CDR + FW) were also equivalent in their ability to block the generation of C5a (Fig. 8). Similarly, bacterial-produced, refolded scFv, containing either human (h5G1.1 scFv (CDR)) or murine (h5G1.1 scFv (CDR + FW)) amino acids at these framework positions, had identical affinities as measured by ELISA (Fig. 5) and inhibited chicken erythrocyte lysis (Fig. 6), porcine aortic endothelial cell lysis (Fig. 7) and C5a generation (Fig. 8) with equivalent potencies. Therefore, although computer modeling suggests the importance of these framework amino acids in CDR canonical structure, amino acid changes at these positions had no effect on the functional activity of the humanized 5G1.1 molecules.

A clinically useful C5 inhibitor must block generation of both C5a and C5b-9, as both of these molecules have

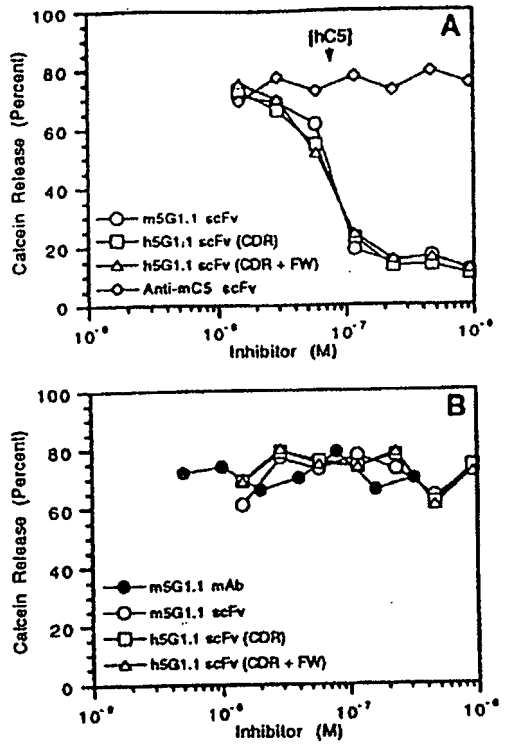


Fig. 7. Protection of porcine aortic endothelial cells (PAEC) from human serum complement by scFv. Calcein-labeled PAEC were incubated with HBSS containing 1% BSA and 0.85 mg/ml purified rabbit anti-PAEC polyclonal antibody for 30 min at 37°C. The cells were washed and incubated with HBSS containing 1% BSA plus 20% human serum (A), or 20% rat serum (B), and the indicated concentrations of inhibitor for 30 min at 37°C. Calcein dye release and retention were determined by measurement of the fluorescence of supernatants and SDS-solubilized cells, respectively, with dye release in the absence of human serum subtracted from all values. Results shown are the average of four to five independent determinations. The arrow denotes the predicted concentration of hC5 in 20% serum.

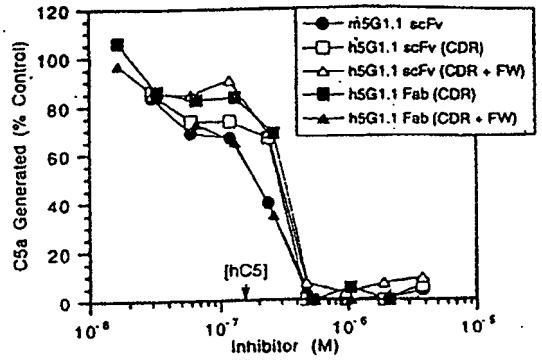


Fig. 8. Inhibition of C5a generation by recombinant Fab and scFv. Supernatants from chicken erythrocyte hemolytic assays performed using 40% human serum, as described in Fig. 5, were assayed for C5a content by ELISA. The C5a concentration obtained in the absence of inhibitor was defined as 100%. Data shown are from a representative experiment of several performed. The predicted hC5 concentration in 40% serum is indicated by the arrow.

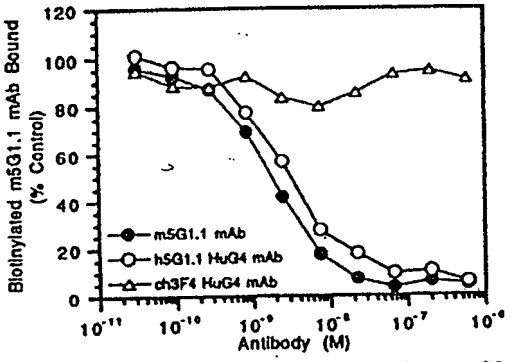


Fig. 9. Inhibition of 5G1.1 mAb binding to human C5 by recombinant humanized h5G1.1 HuG4 mAb. Biotinylated 5G1.1 mAb (0.2 nM), plus the indicated concentrations of unlabeled murine 5G1.1 mAb, recombinant h5G1.1 HuG4 mAb or a negative control recombinant HuG4 mAb (ch3F4 HuG4 mAb), were incubated together for 2 hr at 37°C in microtiter plates coated with human C5. After washing, bound biotinylated 5G1.1 mAb was detected using peroxidase-conjugated streptavidin, with quantitation performed by monitoring hydrolysis of *o*-phenylenediamine dihydrochloride at 490 nm. Absorbance values obtained in the presence of recombinant inhibitor were normalized in each individual experiment, to the value obtained in the absence of competitor. Results shown are mean values of four determinations.

potent pro-inflammatory effects. An scFv has been previously constructed from the variable regions of the monoclonal antibody N19-8 (Würzner *et al.*, 1991). The N19-8 antibody binds to human C5 with an avidity comparable to 5G1.1 (data not shown) and blocks generation of both C5b-9 and C5a. Although the N19-8 scFv has a high binding affinity for human C5 and inhibits the ability of human serum to lyse chicken erythrocytes, it only weakly inhibits C5a generation (Evans *et al.*, 1995). These findings suggest a model in which binding of the N19-8 mAb sterically blocks interaction of C5 with the C5 convertases. The N19-8 scFv may be too small to block cleavage by the C5 convertases, but remains bound to C5b and blocks formation of the C5b-9 complex. In contrast, the h5G1.1 scFv completely blocked generation of C5a when present at only a three-fold molar excess, relative to human C5 (Fig. 8). The 5G1.1 epitope may, therefore, be more directly involved in the interaction of C5 with the C5 convertases, than is the N19-8 epitope. Additionally, the h5G1.1 scFv maintains the ability to block the induction of both C5a- and C5b-dependent pro-inflammatory events in a closed loop model of cardiopulmonary bypass (S. A. Rollins, unpublished results).

Three important attributes of the humanized h5G1.1 scFv suggests its value as a therapeutic. Firstly, it main-

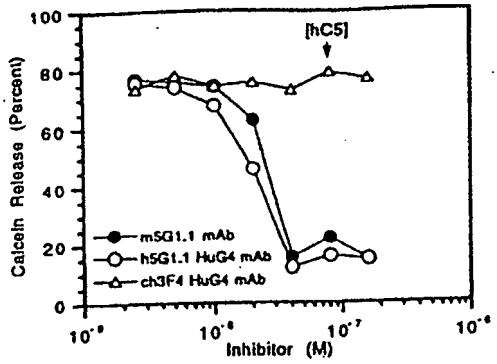


Fig. 10. Protection of porcine aortic endothelial cells from human serum complement by h5G1.1 HuG4 mAb. Calcein-labeled PAEC were incubated with HBSS containing 1% BSA and 0.85 mg/ml purified rabbit anti-PAEC polyclonal antibody for 30 min at 37°C. The cells were washed and incubated with HBSS containing 1% BSA plus 20% human serum and the indicated concentrations of inhibitor for 30 min at 37°C. Calcein dye release and retention were determined by measurement of the fluorescence of supernatants and SDS-solubilized cells, respectively, with dye release in the absence of human serum subtracted from all values. Results shown are the mean of three independent determinations. The arrow denotes the predicted concentration of hC5 in 20% serum.

tains the essential ability to block cleavage of C5 while preserving the immunoprotective properties associated with C3b generation. Secondly, it can be readily produced in large quantities in *E. coli*. Finally, the pharmacokinetics of scFv molecules are well matched to the time frame necessary for complement inhibition in acute settings where complement activation contributes significantly to the inflammatory process, such as cardiopulmonary bypass or myocardial infarction. Clearance of scFv molecules from the blood has previously been found to be extremely rapid with half lives of only a few minutes (Colcher *et al.*, 1990; Milenic *et al.*, 1991). At least two features of scFv molecules are likely to result in a rapid clearance rate, as compared to intact antibodies. Firstly, the prolonged plasma half-life of IgG is mediated through binding of the Fc region to specific "protection receptors", which have recently been demonstrated to be identical to the neonatal intestinal transport receptor (Ghetie *et al.*, 1996; Junghans and Anderson, 1996). Secondly, the small size of the scFv is well below the M_r 60 000 cut off of the glomerular basement membrane, resulting in the loss of the scFv into the urine. However, the binding of the scFv to C5 in the blood results in its incorporation into a high molecular weight scFv–C5 complex of nearly 220 000. Loss of bound scFv through glomerular filtration is, thus, dependent on the dissociation rate of the scFv from C5. Calculations based on the competitive ELISA data (Fig. 5), as well as direct measurements of h5G1.1 scFv (CDR) binding to human C5 by surface plasmon resonance (data not shown), indicated a very high affinity of the scFv for C5 ($K_d = 100$ pM) and a slow dissociation rate ($k_{off} = 1.0 \times 10^{-4}$/sec). This dissociation rate corresponds to an scFv–C5 complex half-life of approximately 2 hr. The 5G1.1 monoclonal antibody recognizes only human C5, precluding a direct assessment of h5G1.1 scFv pharmacokinetics in primates. In contrast, the N19-8 monoclonal antibody, which binds to human C5 with an avidity equivalent to 5G1.1, does bind to rhesus monkey C5. A single 100 mg bolus injection of N19-8 scFv into rhesus monkeys inhibits serum hemolytic activity for approximately 2 hr (Evans *et al.*, 1995b), further suggesting that the h5G1.1 scFv will have *in vivo* kinetic properties sufficient for use in acute clinical settings.

Complement activation has been suggested to be involved in the pathogenesis of several chronic human diseases including allograft rejection (reviewed by Baldwin *et al.* (1995)), systemic lupus erythematosus (reviewed by Mills (1994)), myasthenia gravis (reviewed by Drachman (1994)) and rheumatoid arthritis (reviewed by Morgan (1990)). Recently, it has been shown that prolonged treatment with an antibody to mouse C5 both blocks onset and disease progression in a collagen-induced arthritis model (Wang *et al.*, 1995), as well as inhibits development of glomerulonephritis in NZB/W mice (Wang *et al.*, 1996). As the half-life of the h5G1.1 scFv is likely to be too short to allow for chronic use in humans, a full length humanized antibody was also constructed. The human IgG4 isotype was chosen, as this isotype does not activate human complement (Tao *et al.*, 1993) and there is only one known allotype of IgG4 (Ghanem *et al.*, 1988), precluding the potential development of allo-antibodies in patients. The humanized h5G1.1 (CDR) HuG4 antibody bound to human C5 with a similar avidity as the murine antibody when assayed by ELISA (Fig. 9) and inhibited lysis of porcine aortic endothelial cells as effectively as the murine antibody, with a 1:1 molar ratio of antibody binding sites to human C5 being sufficient for inhibition (Fig. 10). Little information is available on the immunogenicity of CDR-grafted antibodies in humans. Repeated administration of chimeric antibodies containing intact murine variable regions, has induced an immune response directed against the murine variable regions in nearly all trials (reviewed in Khazaeli *et al.* (1994)). In this regard, it is significant that introduction of murine amino acids in the framework regions was not essential for maintenance of high affinity binding to C5. The h5G1.1 antibody is, therefore, likely to be minimally immunogenic in patients.

The recent successes of complement inhibitors in modulating numerous *in vivo* models of inflammation, suggest a great potential for complement inhibitors in the treatment of human inflammatory disease. The development of distinct C5-specific complement inhibitors with pharmacokinetic parameters, tailored for use in either acute or chronic settings, should facilitate the clinical assessment of the role of complement in such diseases.

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CHAPTER 29

Complement

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A LOOK BACK IN HISTORY

In the beginning, i.e., in the second half of the 19th century, research on complement was intertwined with the investigation of humoral immunity and proceeded in parallel with the discovery

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Exhibit I

of important bacterial pathogens (1). Nuttall, Buchner, and others contributed to the first major conceptual advance that a heat-labile fraction in normal serum (i.e., complement) and a heat-stable component of immune serum (i.e., antibody) were necessary for killing bacteria. Buchner named the labile fraction alexin (Greek for "without a name") and postulated an enzymatic mode of action for alexin. Bordet demonstrated a similar mode of action using lysis of erythrocytes by immune serum as a system that was fundamental for his development of the complement fixation test.

The term "complement" itself was coined by Paul Ehrlich in 1899, when he applied his *Seitenkettentheorie* (side-chain theory), which he had developed to explain immune bacteriolysis, to the understanding of hemolysis by sera of sensitized animals (2). "Complement" thus replaced "alexin." Ehrlich thought of complement as a group of factors that would not strictly rely on each other, whereas others (e.g., Bordet) considered complement a uniform substance. The chemical nature of complement was initially considered to be detergentlike or otherwise lipid destroying; the protein nature of complement components became clear much later. From this initial concept of complement (solely) as an effector mechanism of antibody, the entities of C1, C2, and C4 were the first to be differentiated by early biochemical methods until the 1920s. The proteins C3, C5, C6, C7, C8, and C9, however, were still thought of as one factor, as "classical C3."

A major step forward in the understanding of neglected aspects of complement was made by Louis Pillemer in the 1940s and 1950s. He extended previous findings with yeast cells and substances such as cobra venom, which activated complement in the absence of antibody. Pillemer demonstrated that classical C3 was consumed by zymosan, a yeast cell wall mannoprotein, without consumption of C1, C2, and C4 (3). In 1954, he postulated the existence of a serum factor he called properdin (Latin for "destruction-bringing") and understood the properdin system as a second, antibody-independent mode of complement activation (4). Pillemer's hypothesis was heavily criticized initially, and the controversy was ended for some time by his death. However, as the factors of the properdin system were isolated and characterized later on, this concept (now called the alternative pathway of complement activation) became accepted.

The role of C3 as the pivotal factor in both the classical and the alternative pathway and of C5 through C9 forming the terminal complex common to both became clear when classical C3 was biochemically dissected into the individual proteins during the 1960s. Hans Müller-Eberhard and his collaborators identified the C3 protein (according to the current nomenclature) as a major constituent of human plasma and also purified the components C5 to C9 (5). Robert Nelson's and Paul Klein's groups achieved the same for guinea pig C3 (6–8).

The concept of complement-mediated lysis was advanced by Manfred Mayer, who hypothesized that one complex containing all complement components is sufficient to lyse one red cell (9). His "doughnut hypothesis" later summarized results from several groups and postulated that C5b through C9 together formed a channel-like structure in the membrane (the membrane attack complex) (10). Since then, there has been a constant debate whether destruction of a nucleated cell is brought about by the pore character of the membrane attack complex (i.e., by osmotic swelling of the cell) or accomplished through destabilization of membrane integrity. This dispute is still unresolved because there is experimental evidence to support both contentions.

More recently, a second antibody-independent way of complement-activation, the mannan-binding lectin (MBL) pathway (named MBLectin pathway), has been established. MBL recognizes pathogens via their carbohydrate-rich exterior. MASP-2, a serine-esterase associated with MBL and cloned in 1997, is the youngest member in the family of complement proteins to date (11).

Besides research on the complement activation mechanism, there has been early recognition of its proinflammatory effects, such as opsonization and anaphylaxis. At the beginning of the 20th century it was noted that treatment with nonheated serum not only promoted lysis of bacteria, but also their clearance by phagocytosis. This nondestructive effect of serum causing improved uptake of bacteria by phagocytic cells was called opsonization (Greek for "preparation for ingestion"). Due to their heat-labile nature, the underlying serum factors were postulated to be related to complement early on, but proven to be mainly C3 fragments decades later. The existence of receptors for deposited complement components on cells was first postulated in 1953 by Nelson. He demonstrated that bacteria treated with immune serum gain the ability to bind to erythrocytes and called this phenomenon immune adherence. Complement receptors were subsequently identified on phagocytic cells and lymphocytes as well and shown to be specific for fragments of C3.

Heat lability also characterized anaphylaxis, a phenomenon observed after administration of immune complex–treated serum. Like opsonization, anaphylaxis was thus proposed to relate to complement. The generation of a complement-derived anaphylatoxin and another, chemotactic factor was proven by Boyden in the 1960s. These were finally characterized as C3a and C5a.

With the advent of molecular cloning in the early 1980s, the deduced amino-acid sequences were unraveled for all of the known human complement proteins, and the chromosomal locations of their genes have been clarified. The recognition of structural motifs conserved between the individual mosaic proteins allowed the definition of functional groups of components as well as a look back in evolution. Animals genetically deficient for a component as well as transgenic mice have been established and are a field of intense investigation.

Although there have been many contributions on involvement of complement in clinical disorders, it appears that only now, after 100 years of complement research including detailed biochemistry and molecular genetics, the role of complement in a broad spectrum of diseases becomes the focus of research. It concerns, among others, atherosclerosis, Alzheimer's disease, cancer, and, of course, infection and transplantation. It remains an unachieved goal to find ways to interfere with unwanted or excessive complement activation. Clinical use of recombinant proteins such as soluble CR1 or of humanized monoclonal antibodies has started, but to date there are no chemical substances at the disposal of clinical medicine that can selectively and effectively block individual components.

GENERAL OVERVIEW

As suggested by its name, complement serves as an auxiliary system in immunity, both on its own and by interaction with humoral immunity. On its own, it represents a primitive surveillance for microbes, independent from antibodies or T cells. During evolution, it became intertwined with the humoral immune system at multiple levels and now represents a major effector system for antibodies.

The complement system comprises more than 30 plasma or membrane proteins (Tables 1 and 2). Its activation as a whole relies initially on a cascade of proteolytic steps performed by serine pro-

TABLE 1. *Complement components: the plasma proteins involved in activation*

Component	Molecular weight in kDa of the intact protein (of subunits)	Concentration in plasma (μg/mL)
Common to all activation pathways		
C3	185 (α,110; β,75)	1,200–1,300
Alternative pathway		
Factor B	93	200
Factor D	24	2
Properdin (predominant oligomers)	110, 165, 200 (monomer: 55)	25
Classical pathway		
C1q	460 (six subunits with three chains each: A,26; B,26, C,24)	150
C1r	85	50
C1s	85	50
C4	205 (α,97; β,75; γ,33)	300–600
C2	102	20
MBLectin pathway		
MBL (predominant forms)	200, 300, 400 (two to four subunits with three chains of 32 kDa each)	1 (0.01–20)
MASP-1	100	1.5–12
MASP-2	76	nd
Terminal complement pathway		
C5	190 (α,115; β,75)	80
C6	110	45
C7	100	90
C8	150 (α,64; β,64; γ,22)	55
C9	70	60

MBL, mannan binding lectin; MASP, MBL-associated serine protease; nd, not determined.

TABLE 2. *Complement control proteins*

Component	Molecular weight in kDa of the intact protein (of subunits)	Concentration in plasma (μg/mL)
In plasma		
Factor I	88 (50 + 38)	35
Factor H	150	300–450
C1-INH	105	240
C4bp	550 (7 × 70, 1 × 45)	250
S protein (Vitronectin)	84	500
Clusterin (SP-40,40)	70 (35 + 35)	50
Carboxypeptidase N (anaphylatoxin inactivator)	280 (2 × 90, 2 × 50)	35
Related molecules with unclear function		
FHL-1	42	5–20
FHR-1, FHR-2	39/42, 24/29	40–60
FHR-3	55	nd
FHR-4	86	nd

Component (CD number)	Molecular weight (kDa) of the intact protein	Tissue distribution
On cell membranes		
CR1 (CD35)	190 (most common allotype)	(see Table 4)
DAF (CD55)	70	Very wide: peripheral blood cells (except NK cells), erythrocytes, epithelial and secretory cells, endothelial and mesenchymal cells
MCP (CD46)	45–70 (due to glycosylation)	Same as DAF (but not on erythrocytes)
CD59 antigen	18–20	Same as DAF

C1-INH, C1 inhibitor; FHL, factor H–like protein; FHR, factor H–related protein; nd, not determined; CR, complement receptor; DAF, decay-accelerating factor; MCP, membrane cofactor protein.

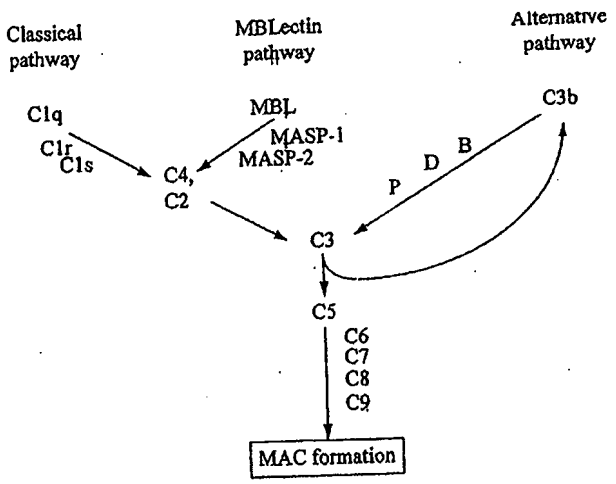

FIG. 1. Overview: the pathways of complement activation.

tease domains in the components involved. Three different pathways of activation have been recognized (Fig. 1), triggered by either target-bound antibody (the classical pathway), by polysaccharide structures of microbes (the MBLectin pathway) or by recognition of "foreign" surface structures by complement itself (the alternative pathway). All three merge into the pivotal activation of C3 and, subsequently, of C5. In the common terminal pathway, further complement components are activated in a nonproteolytic manner and assembled into the membrane attack complex (MAC), which can directly bring about lysis of a microbe.

Such a powerful machinery needs safe, redundant control mechanisms. Therefore, about half of the complement components serve for controlling the critical steps in activation, especially those dealing with C3b generation. In addition to direct killing of microbes by the MAC, complement recruits other branches of the host's defense system (Fig. 2). Opsonization, the coating of microbes with C3 fragments, leads to their uptake into phagocytic cells via complement receptors. The solubility of immune complexes and the immunogenicity of antigens are improved by attachment to C3 fragments. On the other hand, cells of the unspecific defense system (like neutrophils, macrophages, or mast cells) become stimulated by the anaphylatoxins, small peptides generated in the course of proteolytic complement activation.

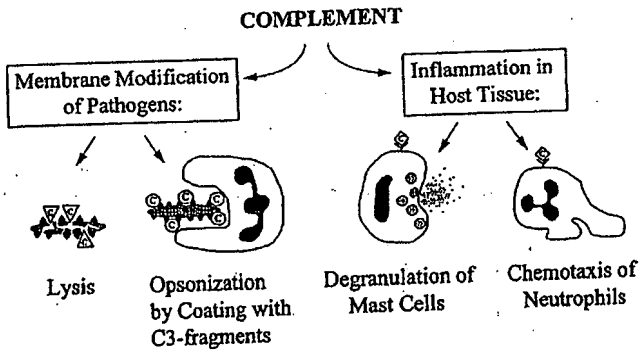

FIG. 2. Overview: the contributions of complement activation to infection control.

Due to historical reasons the components of the classical and terminal pathways are numbered from C1 to C9, with the biochemical reaction sequence being C1-C4-C2-C3-C5-C6-C7-C8-C9. Additional proteins operating in the alternative pathway are called factors and distinguished by letters (factors B, D, H, I, P). Up to C5, activation involves a proteolytic cleavage step, liberating smaller fragments from C2 through C5, which in part exert biologic effects. The larger fragments remain bound in a complex required for the next activation step. By convention, the smaller fragments are denoted by the letter a (e.g., C3a, C5a), the larger by b (e.g., C3b), with the notable exception being C2 (C2a is the larger, active fragment). For C3 and C4, inactivation of C3b or C4b yields smaller fragments (not participating further in complement activation), denoted C3c, C3d, etc. The activation products must not be confused with the terms denoting the protein chains in molecules consisting of disulfide-linked chains, such as C3, C4, and C5, which are indicated by Greek letters (e.g., C3α). Still different is the classification of the two allelic forms of the C4 gene, C4A and C4B. The encoded isotypic proteins C4A and C4B differ in characteristic amino acid residues, which determine the preference of the thioester bond to react with either amino groups (C4A) or hydroxyl groups (C4B) on acceptor molecules. The nomenclature of C3 convertases used here is according to Müller-Eberhard (12). The membrane proteins in the complement system are named under several points of view: either by the function they exert (e.g., decay accelerating factor [DAF]) or by using the cluster of differentiation (CD) system (e.g., CD55 for DAF). The four complement receptors are also simply numbered consecutively (CR1 to CR4).

BIOSYNTHESIS OF COMPLEMENT: LOCATION AND REGULATION

The liver is the major site of production for complement proteins. About 90% of the plasma complement components are synthesized in the liver (13). Only few components have their origin predominantly outside of the liver: C1 in the intestinal epithelium and monocytes/macrophages, and factor D in adipose tissue. C7 of hepatic origin was found to contribute less than 60% to plasma C7 (14,15). Bone marrow–derived cells, particularly granulocytes, apparently represent a major source of plasma C7 (15,16). The main source for plasma properdin has not yet been identified (13).

In addition to the liver, complement component biosynthesis has been detected in many other organs and cell types, such as monocytes/macrophages, endothelial cells, lymphocytes, glial cells, renal epithelium, reproductive organs and many others. Notably, production of virtually all components has been observed in monocytes/macrophages and, interestingly, in astrocytes (17). The contribution of extrahepatic complement production has not been clearly defined: as for astrocytes or other glial cells, they are the only source for complement beyond an intact blood–brain barrier. Hence, the role of complement in the brain is an emerging field of interest in several, primarily noninfectious diseases. Macrophages, by their presence in an activated state at sites of infection, may add to the locally effective levels of complement.

Complement production is augmented in the acute-phase response that follows tissue injury. This pertains to most components, although the extent of induction varies substantially (from about three- to 50-fold). The main common transcriptional inducer

of complement genes is interferon (IFN)-γ, with other important acute-phase mediators being interleukin (IL)-1– and IL-6–type cytokines (IL-1α, IL-1β, tumor necrosis factor [TNF]-α, IL-6, IL-11, and others) (18).

Membrane-anchored complement regulators are expressed on a variety of tissues (13). Even complement receptors are widely distributed, although expression may be weak and noticed only upon cell activation.

GENETIC FAMILIES AND STRUCTURAL MOTIFS AMONG COMPLEMENT COMPONENTS

Proteins Endowed with an Internal Thioester

C3, C4, and C5 are proteins considered to be evolutionarily derived from one ancestral protein. Upon proteolytic cleavage at a conserved site during complement activation, they undergo a gross conformational change associated with the exposition of several new epitopes and (in C3 and C4) the ability to covalently bind to other molecules. This capability is linked with the formation of an internal thioester in the native molecule between a glutamyl residue and a cysteine two residues apart (Fig. 3) (19). This thioester is present in C3, C4, and the related α2 macroglobulin, but has been lost in C5 during evolution. Hidden in the native proteins, the thioester is exposed upon activation to react with the NH_2 or OH residues of surrounding molecules. The two isotypic forms of C4, C4A and C4B, differ by the presence and absence, respectively, of a histidine 115 amino acids downstream, which acts as the catalyst for the formation of ester bonds. Hence, C4B and C3, which behaves similar to C4B, preferentially form ester bonds with OH residues, whereas C4A forms amide bonds with NH_2-groups (19). It has been suggested that formation during biosynthesis needs the presence of chaperone molecules that may be different for the individual thioester proteins (19).

Proteins with Short Consensus Repeats

The regulator of complement activation (RCA) gene cluster comprises the genes for factor H and related proteins of the factor H family, for C4bp (several loci), and for DAF, CR2, CR1, and MCP (20). The RCA proteins consist of four to 34 short consensus repeats (SCRs) (Fig. 4), eventually in addition to short transmembrane and intracytoplasmic parts (CR1, CR2, MCP) or glycosylphosphatidylinositol (GPI) anchors, as in the case of DAF (21). The consensus SCR is a globular domain of about 60 amino acids, with distinct conserved residues, e.g., tryptophane, prolines, or, most importantly, four cysteines that form two disulfide bonds (Cys_1 to Cys_3 and Cys_2 to Cys_4) (22). The RCA proteins are elongated in shape, with CR1 and CR2 extending 90 and 28 nm, respectively, from the cell membrane (Fig. 4). Electron microscopy has shown the plasma protein factor H to have an elongated, hairpinlike structure.

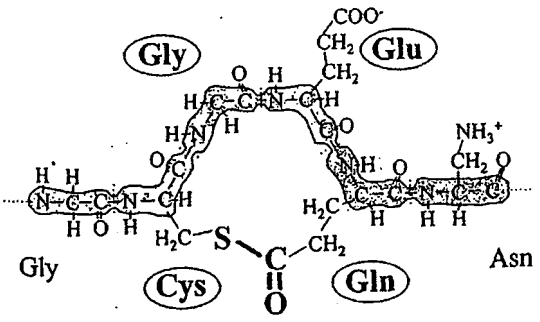

FIG. 3. The thioester region of human C3: the backbone of peptide bonds (*shaded*) and the four amino acids forming the thiolactone ring (*encircled*). The thioester bond is shown in bold print.

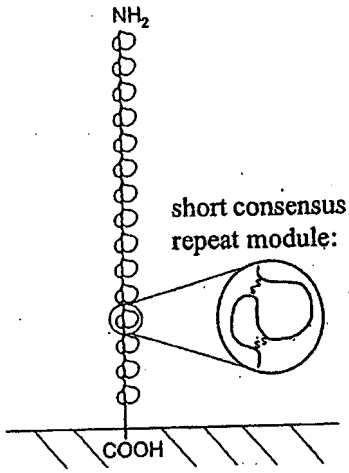

FIG. 4. The structure of the SCR and of SCR-based complement regulators (e.g., for CR2).

The RCA gene cluster is thought to have evolved from one ancestral prototypic SCR by duplication and gene conversion events as a family of genes for proteins controlling C3 and C4 activation (23). Interestingly, though, binding of activated C3/C4 can be attributed only to a few distinct SCRs in each RCA member (24). Few SCRs are present in mosaic proteins such as factor B, C2, C1r, C1s, MASP-1, MASP-2, C6, and C7, all of which interact with C3/C4/C5. SCRs are also found in noncomplement proteins as factor XIIIB of the clotting system, IL-2 receptor α chain (CD25) and selectins. Because each SCR is usually encoded by a separate exon, these combinations of domains can be seen as the result of exon shuffling.

Modified Serine Proteases

Serine proteases are crucially involved in the early, amplifying steps of complement activation. Serine protease domains are present in C1r, C1s, MASP-1, MASP-2, C2, factor B, factor I, factor D, and many other noncomplement enzymes (e.g., trypsin).

Thrombospondinlike Repeats Containing Proteins

A 30–amino acid module also found in the extracellular matrix protein thrombospondin is present in constituents of the terminal pathway and in properdin (25). C6 has three thrombospondinlike repeats (TSRs); C7, C8α, and C8β all have two TSRs; and C9 possesses a single TSR module. Six tandem TSRs are found in prop-

erdin. These proteins have amphiphilic character, allowing them to act in plasma and on lipid membranes, which is the important feature for MAC formation.

Members of Other Structural Families

Serpins comprise proteins acting as serine protease inhibitors. Among the many serine proteases active in the complement system, only C1s and C1r are inhibited by a serpin, namely C1 inhibitor (C1-INH).

MBL is a collectin, a lectin with structural resemblance to collagen in the stalk parts of its subunits. Although C1q does not bind to carbohydrates, it is structurally related to the collectins, primarily MBL. Both form collagenous and globular domains and share the feature of assembly from several identical subunits.

C9, apart from including a TSR, is homologous to perforin, the pore-forming protein of cytotoxic T cells and natural killer cells. The terminal pathway components also comprise one low density lipoprotein-receptor domain and one epidermal growth factor (EGF) module.

CR3 and CR4 belong to the large integrin family of heterodimeric proteins. Their β chain is of the β2-integrin type, which is also present in leukocyte function antigen-1. Integrins are mainly involved in cell–cell and cell–matrix interactions. CR3 and CR4 have similar properties as well.

The receptors for the anaphylatoxins C3a and C5a belong to the G-protein–linked receptors (seven-pass transmembrane receptors), which cross the cell membrane seven times with α-helical stretches and are coupled to (intracellular) G proteins.

COMPLEMENT ACTIVATION: THE PIVOTAL ROLE OF C3 ACTIVATION

Activation of C3 by cleavage to C3b is the pivotal reaction in the activation cascade (Fig. 5). This reaction is common to all three activating pathways and catalyzed by two different C3 convertases. Although some other proteases (like plasmin) or toxins (e.g., cobra venom factor) can activate C3, the C3 convertases are the only physiologically relevant effectors. Of all complement components, C3 is present in the highest concentration (1 to 1.4 mg/ml plasma) and is one of the most abundant plasma proteins. Due to the presence of an intramolecular thioester, C3, together with its closest relative C4, is the only component able to form covalent bonds with various targets.

It appears that the complement system has evolved around the capability of this protein (or its ancestor) to covalently bind to other molecules. An ancestral C3 protein might have resembled the thioester-containing protease inhibitor α2 macroglobulin, which binds covalently to various proteases if they cleave α2 macroglobulin and hence induce its active conformation.

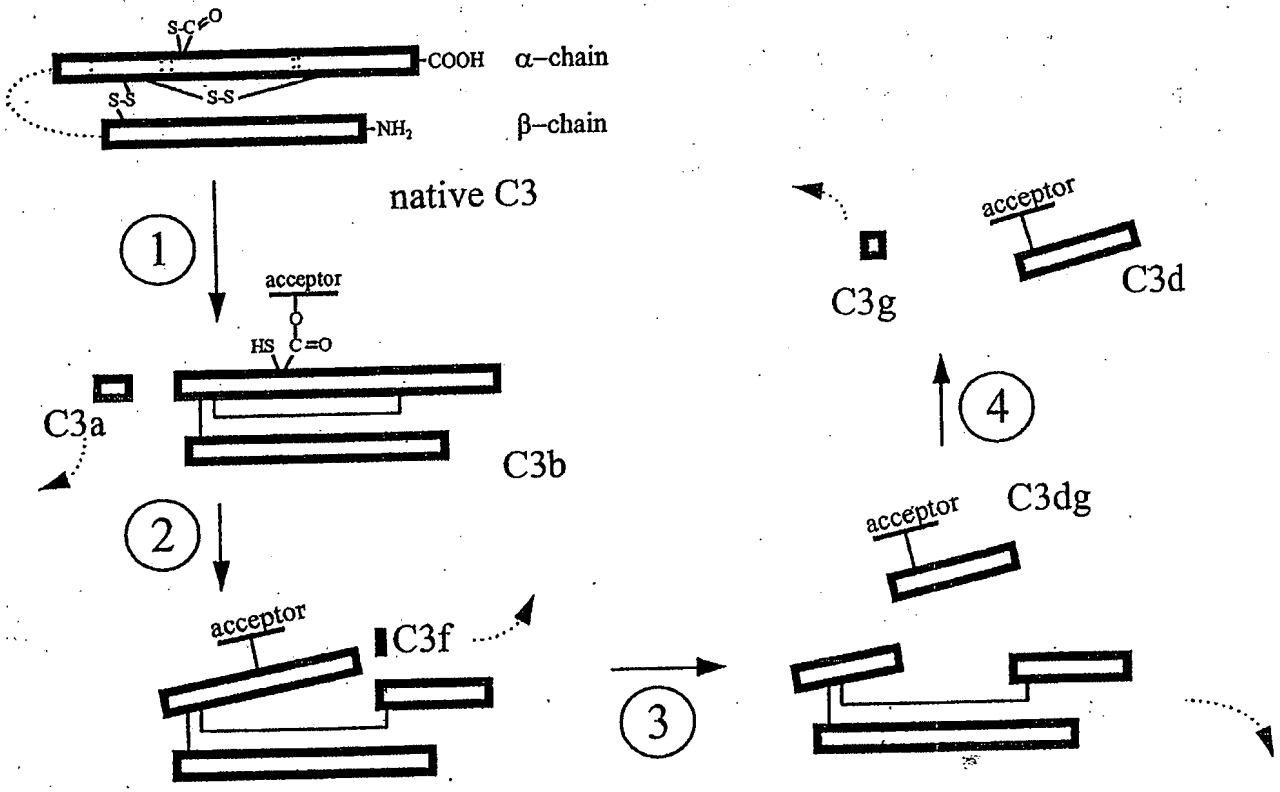

FIG. 5. Activation, inactivation, and degradation of C3. (1) Activation of native C3 by C3 convertases yields C3b (bound to an acceptor molecule) and C3a. (2) C3b is inactivated to iC3b by factor I and a cofactor that cleaves off C3f. (3) iC3b is further degraded by factor I and CR1 to C3dg and C3c. (4) Acceptor-bound C3dg is trimmed by plasma proteases to C3d.

Four functional entities act on C3 and its derivatives:

1. The C3 convertases, two homologous enzymatic complexes (C3b,Bb and C4b,2a, respectively) that consist of an activated serine protease and C3b or C4b; they activate native C3 by cleaving it into C3a and C3b.
2. Factor I, a plasma serine protease specific for C3b (and C4b); it inactivates C3b (and C4b) by cleaving it into iC3b (and iC4b). Factor I requires a cofactor.
3. Proteins of the RCA family consisting of four to 34 SCRs; they negatively regulate C3 (and/or C4) activation by disintegrating the convertases and serving as cofactors for factor I.
4. The receptors for fragments of C3, comprising genetically unrelated proteins such as integrins, seven-pass transmembrane proteins, or RCA family members; they exploit the remnants of C3 activation for the activation or attraction of cells (e.g., in opsonophagocytosis).

Through the action of either C3 convertase, the native C3 molecule is cleaved at a specific arginine residue in the α chain into C3a and C3b. The peptide C3a (77 amino acids) is a potent anaphylatoxin and exerts its effects more distant from the site of C3 activation. The major part, nascent C3b, acts within its life span of 60 microseconds. Nascent C3b immediately changes its conformation and exposes the buried internal thioester bond. Via the now highly reactive thioester, nascent C3b is enabled to bind covalently to proper nucleophils, either OH or NH_2 groups of any surrounding molecule (termed acceptor molecule), including H_2O molecules.

THE ALTERNATIVE PATHWAY

Phylogenetically the oldest of the C3-activating pathways, the alternative pathway represents the first line in defense against invading microorganisms (Table 1 and Fig. 6). It can be activated and amplified instantaneously in the presence of foreign (nonself) material. The alternative pathway defines "foreign" by other criteria, as do antibodies, and hence represents a primitive immune system on its own. An ancestral alternative pathway system was probably present about 500 million of years ago and is found in the most primitive vertebrates like lamprey and hagfish (23). Circumstantial evidence supports the existence of a primitive alternative pathway or a C3 analog in nonvertebrates like the horseshoe crab or even insects, possibly linked to an ancestral humoral immune system based on recognition of (foreign) carbohydrates.

After the evolution of the immunoglobulin system, it became as a new trigger to the old effectors through the classical pathway.

The proteins participating in the alternative pathway are C3 (and C3b) and the factors B and D. These proteins can establish a positive feedback loop of C3 activation (the C3b amplification loop). Properdin (factor P) favors the amplification loop by stabilizing the convertase C3b,Bb. Proteins controlling the alternative pathway are factor I (together with its cofactors factor H, CR1 and MCP) and DAF.

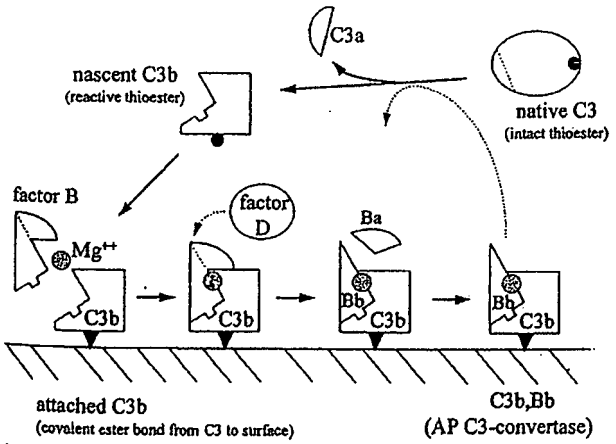

FIG. 6. C3b amplification by the alternative pathway (AP). By action of the alternative pathway, C3 convertase C3b,Bb native C3 is activated, changes its conformation, and exposes the reactive thioester (●). Some of the nascent C3b attaches covalently to an activator surface via the thioester (▼), where it associates with factor B in the presence of Mg^{2+}. Bound factor B is cleaved by factor D, Ba is released, and Bb remains bound to C3b, thus forming a new AP C3 convertase.

Initiation by iC3

A longstanding conceptual problem was to explain generation of the first C3b. From in vitro experience rather then from plasma level measurements, the concept of alternative pathway was formulated that relies on the initiation by inactive C3 (iC3 or C3b-like C3).

This concept is based on a continuous background turnover of native C3 into iC3 (termed "tickover of C3"). iC3 results from spontaneous reaction of the internal thioester bond with water [hence its former name C3(H_2O)]. Thus, iC3 represents uncleaved, but hemolytically inactive C3 with C3b-like conformation. iC3 forms in plasma at a constant low rate, and its actual presence in plasma has been proved years after being postulated and was shown to be 0.5% of the amount of native C3 (26).

iC3 can associate with factor B in a Mg^{2+}-dependent reaction. In this complex, the zymogen factor B is accessible to cleavage by factor D. The enzymatically active Bb fragment remains attached to iC3, thus forming iC3,Bb, the initial C3 convertase of the alternative pathway. It is thought that this initial convertase is constantly formed in the fluid phase but has a very short half-life. iC3,Bb is quickly disassembled by factor H, and iC3 is readily cleaved by factor I in analogy to C3b (Fig. 5). Nevertheless, this would still allow the generation of some nascent C3b molecules that could attach at random to nearby plasma or surface molecules.

The fate of such a surface-bound C3b molecule would be determined by the activator or nonactivator character of the surface. Whether a particle is an activator (evoking massive C3 activation and C3b deposition on its surface) or a nonactivator (effectively limiting this reaction) is determined by the relative affinities of bound C3b to factor H, the negative regulator, and factor B, the positive regulator of the alternative pathway. The ratio of factor H to factor B affinity is mainly influenced by the decreased affinity of factor H to activator surfaces (about tenfold less), whereas factor B affinity is similar to activator or nonactivator surfaces.

Amplification of C3b by the Alternative Pathway on Activator Surfaces

With a first C3b molecule randomly attached to an activator surface, however, amplification of C3b proceeds rapidly (Fig. 6).

First, factor B associates with C3b in the presence of Mg^{2+} and is activated by factor D, a serine protease present in plasma in minute amounts. Factor D is brought into its active conformation through recognition of its substrates, C3b,B or iC3,B (27). After generating C3b,Bb and releasing Ba, factor D returns to its inactive state. The surface-attached C3b,Bb activates further C3 molecules, and some of the new nascent C3b will attach again to the surface. C3b,Bb remains active as long as Bb remains bound to C3b, and properdin stabilizes the convertase against decay by binding to both Bb and C3b.

Inactivation of C3b on Nonactivator Surfaces

On nonactivator surfaces such as host cell membranes, the binding of factor H is promoted by its affinity to negatively charged residues like multiple sialic acid molecules. Their presence on the carbohydrate part of glycoproteins allows C3b bound to host cells to be quickly bound to factor H and subsequently cleaved by factor I. Factor H also can dissociate C3b,Bb enzymes, which have eventually formed on nonactivators or which are present in the fluid phase (see section on "Control of the Complement System" and Fig. 10).

THE CLASSICAL PATHWAY

Proteins of the Classical Pathway

The proteins forming the activation cascade of the classical pathway comprise C1, C4, C2, and C3, in that order (Table 1 and Figs. 1 and 7). C1 inhibitor (C1-INH), C4-binding protein (C4bp), CR1, factor I, DAF, and MCP function as control proteins.

C1 is a large molecule (MW = 750 kDa) consisting of one C1q molecule noncovalently associated with two C1r and two C1s molecules (Fig. 7). Calcium ions are required for formation of this stable complex, $C1q(C1r)_2(C1s)_2$. In plasma, about 70% of the C1 components are present in C1 complexes at a given time. The C1q protein is assembled from six identical subunits, each of which consists of three homologous chains (A, B, and C). The chains form a globular domain at one end, a neck portion, and a stalk part where the three α-helices are twisted around each other and, like in the collagen molecule, form a coil. The six subunits are held together in their collagen-like parts. This appearance of C1q is often likened to "a bouquet of six tulips." The globular domains of C1q bind to the Fc portion of immunoglobulins. A similar overall structure applies to MBL.

C1q interacts with C1r and C1s in its stalk part. The $C1r_2C1s_2$ tetramer has been shown by electron microscopy to form a linear chain of subcomponents (28). Each C1s and C1r possesses a serine protease domain (catalytic domain) and a contact domain. Before activation, all four catalytic domains are placed inside the cone-shaped stalk part of C1q (Fig. 7).

Complement Activation via the Classical Pathway

Physiologically most important, activation of C1 is initiated by its binding to antigen-bound IgG or IgM. Nevertheless, other triggers of C1 activation besides immunoglobulins have been found and include bacterial lipopolysaccharide (LPS), polyanionic compounds, myelin, the acute-phase reactant C-reactive protein, and some viruses (e.g., human immunodeficiency virus [HIV]-1). When binding to immunoglobulin, C1q recognizes the Fc region, which has undergone conformational alteration upon binding to

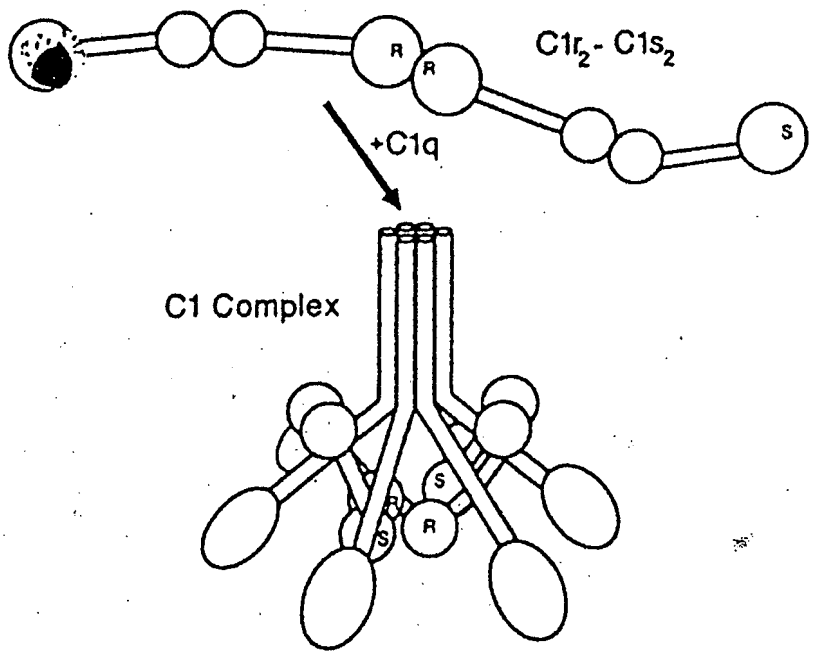

FIG. 7 The C1 complex. The model for the C1 complex proposes that the folding of the rodlike C1r2-C1s2 around the arms of C1q causes the catalytic domains of C1s to contact the catalytic domains of C1r.

antigen. C1q must at least bind with two of its six C-terminal globular domains, one IgM (having five Fc regions per molecule) or at least two IgG molecules are required to trigger complement activation, and they must be in sufficient proximity (not more than 40 nm apart). Therefore, with IgG the activation of C1 will only be effective on surfaces with a critical density of bound antibodies. Among human IgG subclasses, the potential for C1q binding increases in the order IgG4 < IgG2 < IgG1 < IgG3.*

The positions of the serine esterase domains of the two C1r relative to each other change due to conformational alteration of C1q after immunoglobulin binding. This allows for reciprocal cleavage of the C1r molecules. Activated C1r then cleaves (activates) C1s, which is the enzyme activating C4 and C2. Cleavage of C1r and C1s does not liberate proteolytic fragments.

C1s and C1r are tightly controlled by C1-INH in the unbound C1 molecule, which tends toward autoactivation. Activated C1r and C1s are rapidly inactivated by covalent binding of C1-INH to both in a stoichiometric relationship [yielding two C1rC1s(C1-INH)$_2$ molecules per C1]. Nevertheless, although the half-life of active C1 is thus very short, one active C1 molecule can cleave about 35 C4 molecules due to its low K_m value and the high plasma concentration of C4 (29). C4 is cleaved into the short C4a fragment, which exhibits low chemotactic activity, and the large C4b, which undergoes a gross change in conformation. As a result, the internal thioester region within C4b is exposed and forms covalent amide or ester bonds with surrounding molecules (proteins, carbohydrates, water). These reactions take place within microseconds (19). Most of the nascent C4b gets lost by reacting with water, but about 5% of C4b becomes covalently attached to the particle surface in the immediate vicinity of the focus of the activating immunoglobulin–C1 complex (30). In this way a cell or particle surface becomes covered with C4b clustered around central C1 molecules.

Due to its lower plasma concentration, activation of C2 proceeds less effectively than C4 activation (12). About four C2 molecules are activated during the life span of one active C1 molecule. C2 compensates for this by forming an Mg^{2+}-dependent complex with C4b. In this complex, C2 is accessible for cleavage by C1s into C2a (larger fragment remaining associated to C4b) and C2b (liberated smaller fragment exhibiting kinin activity). Free C2 is much less likely to be cleaved by C1s. C2a is the enzymatically active fragment in C4b,2a, the classical pathway C3 convertase. It is active only as long as it is associated with C4b, and once dissociated, it cannot bind to C4b again. The C3 convertase activates C3 and contributes to C5 activation, which then initiates the terminal pathway.

Role of the Classical Pathway

The classical pathway is the phylogenetically youngest among the three activation pathways. It developed after the emergence of the immunoglobulin system in the vertebrates as a potent effector mechanism for humoral immunity. The formation of specific antibodies requires several days, during which defense against infection has to rely on natural immunity: the alternative and MBLectin pathway, opsonization and phagocytosis, other plasma defense proteins (CRP, α2 macroglobulin), and NK cells. By triggering C3 activation via the C1-C4-C2 cascade, Ig combines the effective C3b amplification loop of the older alternative pathway and the formation of membrane attack complexes with a much more potent release mechanism.

THE MBLECTIN PATHWAY (OR LECTIN PATHWAY)

The concept of the MBLectin pathway of complement activation has emerged only recently (see Fig. 1). Its main constituent is the plasma protein mannan-binding lectin (also called mannose-binding lectin), MBL (31).†

MBL is a protein of the collectin family, meaning that it comprises collagenous structures (α-helical parts of three subunit chains twisted around each other to form a coiled-coil bundle) and also functions as lectin. This means it recognizes specific carbohydrate residues by the C-terminal globular part of each subunit chain. MBL is a C-type lectin that binds to its preferred sugars dependent on Ca^{2+}. Among other collectin family members are the lung surfactant proteins SP-D and SP-A and the bovine serum protein conglutinin. The overall structures of the individual collectins are quite different, with MBL resembling C1q in its "bouquet of tulips" appearance. Although C1q is not further included in the collectin family, C1q and the collectins share several features: the subunit structure with its collagenous part and its C-terminal globular domains and the assembly of several subunits by disulfide bonds into the final molecule.

MBL has originally been characterized in other species (33). Human MBL is present in plasma as a mixture of oligomers of its subunit with trimers/tetramers and pentamers/hexamers, constituting approximately 80% and 15% of the pool, respectively (34). MBL levels increase during an acute-phase response by about threefold, which is a less strong induction than seen with several other acute-phase proteins (35). Normal plasma levels also differ substantially between individuals (10 μg/ml to 20 μg/ml; see Table 1) and are genetically determined (36,37). Additionally, distinct allelic forms of MBL are known to be differently effective with respect to complement activation. It is thought that these quantitative and qualitative differences influence predisposition to infections. In fact, deficiency in MBL had been recognized as a functional defect of serum in some patients much earlier and termed defective yeast opsonization (38). The underlying molecule and mechanism, however, was unraveled only recently, when MBL was shown to activate the classical pathway (39).

MBL does so after binding with its globular heads (the carbohydrate recognition domain [CRD]) to sugar residues like N-acetylglucosamin or mannose. Because the ligand affinity of the individual CRD is low (K_d of approximately 10^{-3} M), MBL will only bind if several of its CRDs become attached to oligo- or polysaccharide residues. Such repetitive carbohydrate patterns are often encountered with LPS or other microbial surface structures. The distance between the individual CRDs is big enough not to allow binding of one MBL to a single mammalian glycoprotein (31). On the other

*Some mouse immunoglobulin subclasses can also activate human complement, which is exploited for selective killing of human cells by monoclonal antibodies and human serum (e.g., to achieve pure preparations of lymphocyte subsets). However, the isotype order is different from that of humans.

†Because MBL is the only lectin to activate complement, the term "MBLectin pathway" was suggested by C. Janeway as a substitute for "lectin pathway." The former terms for MBL, mannan-binding protein or mannose-binding protein (MBP), were proposed to be discontinued to avoid confusion with maltose-binding protein or myelin basic protein (32).

hand, several pathogens have been shown to bind MBL, e.g., *Salmonella, Listeria, Neisseria* species or *Candida albicans,* and *Cryptococcus neoformans,* whereas the presence of a bacterial capsule significantly impairs MBL binding (31). A conformational change accompanying ligand binding leads to activation of two MBL-associated serine proteases, MASP-1 and MASP-2 (11). MASP-1 and MASP-2 are both homologous to C1r and C1s, emphasizing the analogy between MBL and C1q. Active MASP-2, like C1s, activates C4 and leads to C1-independent formation of classical pathway C3 convertase C4b,2a. Control of the MBL pathway seems to be exerted through α2-macroglobulin and C1-INH, both of which can bind covalently to the activated MBLectin/MASP complexes (40,41). In contrast to the C1 complex, very little is known about the sites involved in complex formation between MBL and MASP-1/MASP-2 to date.

Complement activation is not the only contribution of MBL to host defense. Bound MBL is recognized by the collectin receptor (42). Because the affinity of MBL to the collectin receptor is low, clustering of the receptor on the cell membrane and the presence of multiple ligands are required for a strong interaction. Whether this collectin receptor actually mediates the opsonic effect on phagocytes is still a controversial issue.

ACTIVATION OF C5

All three pathways of complement activation unite, as outlined, in the activation of C3 by two different C3 convertases (Fig. 8). These same molecular complexes are also used for the next activation step in the cascade, the cleavage of C5, but they need an additional C3b molecule covalently deposited immediately next to them. This C3b acts like an anvil for C5: it interacts with C5 and presents C5 in the correct conformation for cleavage by the C2a part or the Bb part of the respective C3 convertase. Hence C3b,Bb,C3b and C4b,2a,3b constitute the two different C5 convertase complexes. Both require Mg^{2+} ions. Cleavage of C5 in the α chain generates the 11-kDa C5a peptide and the larger fragment C5b. C5a is a very potent chemoattractant peptide that acts distantly from the site of complement activation. C5b is the starter molecule for the formation of the membrane attack complex.

THE TERMINAL COMPLEMENT PATHWAY

The terminal complement pathway is the same whether activation is initiated via the classical, alternative, or MBLectin pathway (see Figs. 1 and 8). After cleavage of C5 by either the classical or the alternative C5 convertase, the terminal complement components C6, C7, C8 and C9 are sequentially, but nonenzymatically, activated, resulting in the formation of the terminal complement complex (TCC) (43).

TCC can be generated on a biologic target membrane as potentially membranolytic MAC, or in extracellular fluids as nonlytic SC5b-9 in the presence of S protein (also called vitronectin). Both forms consist of C5b and the complement proteins C6, C7, C8, and C9. After cleavage of C5, C5b undergoes conformational changes and exposes a binding site for C6. The ability of C5b, staying near the C5 convertase on the target surface, to bind C6 decays rapidly, but once bound, C5b6 forms a stable bimolecular complex. C5b6 binds C7, resulting in the exposure of membrane binding sites and incorporation into target membranes. If C7 concentrations near the site of complement activation are limiting, the stable bimolecular C5b6 complex dissociates from the C5 activating complex and accumulates in solution. In the presence of C7, fluid phase C5b-7 is formed that will not necessarily stay soluble because it has a transient ability to secondarily attach to membranes and initiate lysis, a process called reactive lysis (44). Both the membrane-bound C5b-7 complex as well as the fluid phase C5b-7 complex are capable of binding C8. C8 consists of three nonidentical polypeptide chains: the α and γ chains are covalently linked by a disulfide bond, and the β chain is attached by noncovalent forces. Nascent C5b-7 binds to C8 β via C5b. The C8 γ chain does not appear to

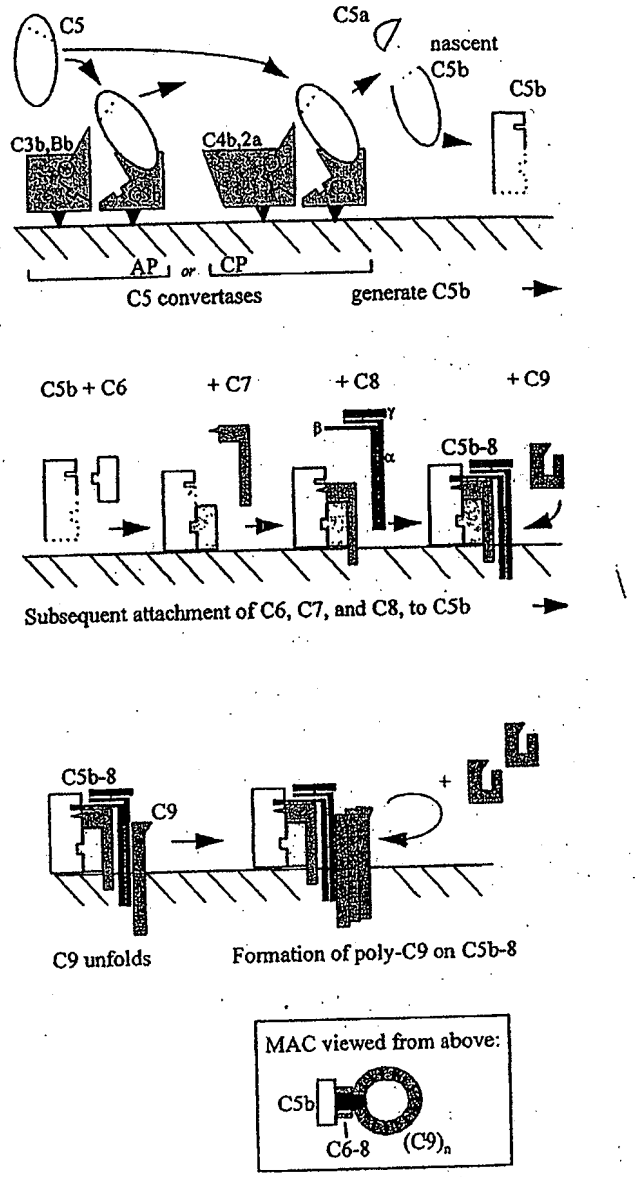

FIG. 8. Activation of C5 and terminal complement pathway. C5 is activated by C5 convertases of the classical or alternative pathway. Nascent C5b interacts sequentially with C6, C7, and C8 and attaches to lipid membranes. As a last step, C9 polymerization on C5b-8 completes the MAC.

have a function in complement lysis, probably because it does not lie adjacent to the membrane but faces the extracellular plasma (Fig. 8).

Although some lytic activity is expressed by the C5b-8 complex, efficient lysis is dependent on an interaction with C9, facilitated by the α-moiety of C8. C5b-8 acts as a polymerizing agent for C9. The first C9, after binding to C5b-8, undergoes major structural changes, enabling formation of an elongated molecule, and allows binding of further C9 molecules and insertion of C9 cylinders into the target membrane (Fig. 8). Whereas only one molecule of each terminal component C5b, C6, C7, and C8 is involved in TCC formation, the number of C9 molecules varies from one to three in the fluid phase and from one to 12 in the membrane-bound form, although polymers containing up to 15 C9 molecules are also possible, provided sufficient amounts of C9 are available. Due to the different number of C9 molecules involved, the tubular structure is not homogeneous. In solution, C9 is also capable of polymerizing with itself without binding to C5b-8, and this tendency toward polymerization can be increased by the presence of metal ions.

The precise mechanism of terminal complement-mediated cytotoxicity after insertion of C9, however, remains unresolved. Currently, two popular hypotheses that do not necessarily exclude each other have been proposed and vigorously defended. According to one model, the polar domains of inserted complement proteins, particularly C9, cause local distortion of the phospholipid bilayer, resulting in leaky patches (45). The other theory postulates that the terminal complement proteins form a hydrophilic channel (pore) through the membrane with consequent disruption of the cell (46).

Membrane perforation by complement is not a unique feature. Perforin, which is contained in the cytoplasmic granules of cytotoxic T-lymphocytes and natural killer cells, is capable of polymerizing on target membranes, thereby forming transmembrane channels. It shares a strong homology with C9. Thus, after antibodies or T cells have identified a target, unspecific destructive forces (i.e., C9 or perforin) take action.

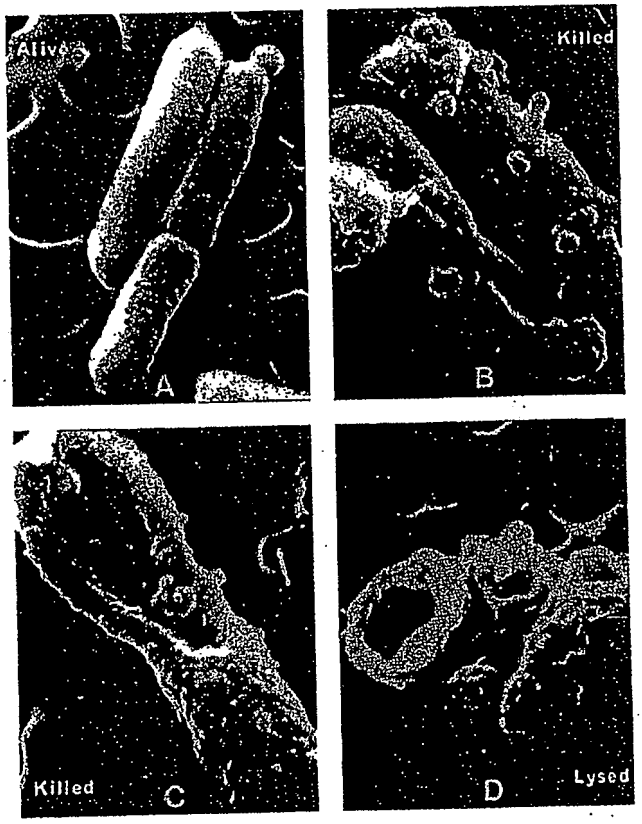

FIG. 9. The structure of *Escherichia coli* as seen in scanning electron micrographs before and after killing by complement. A: Intact bacteria. B and C: Bacteria killed by purified complement proteins. D: Bacteria killed by the combined action of complement and lysozyme (a circulating enzyme that helps degrade bacterial cell walls). Reprinted with permission (47).

Biological Properties of the Terminal Complement Complex

The TCC has been implicated in a large number of diseases because of its presence in diseased tissues or its elevated levels in the blood, although it is usually not clear whether the detected TCC has a significant pathogenic role. However, its lytic properties are important in host defense against bacterial and viral infections (Fig. 9).

On nucleated cells that are not unequivocally identified as non-self, complement activation is often sublytic (48). The term "sublytic" is of a quantitative, not qualitative, nature (i.e., the number but not the structure of TCC complexes is different. Sublytic attack offers some protection to the cell because it can withstand single (and erroneous) attacks, unlike erythrocytes, which are lysed by a single hit. Furthermore, previous sublytic effects exerted on nucleated cells even protect from further, otherwise lytic doses, favoring those cells that are constantly in contact with complement, as host cells (49). Sublytic attack not only protects host cells, but it also stimulates their protein neosynthesis and arachidonic acid metabolism and activates polymorphonuclear leukocytes. In particular, sublytic TCC on nucleated cells transiently increases intracellular Ca^{2+} and activates protein kinase C and guanine nucleotide-binding regulatory proteins (G proteins) (50). It also has the potential to induce procoagulant and proinflammatory activities (48,51). Likewise, the presence of TCC on the surface of viable immune cells suggests a modulating role in the physiology of cells to which it attaches (52). Thus, the main biologic functions of the terminal complement cascade as an important humoral effector arm of host defense thus extend far beyond those originally described. Whether SC5b-9 represents simply the inactivated form of the TCC or whether it plays a role in immune defense remains controversial.

CONTROL OF COMPLEMENT ACTIVATION

As a potentially self-damaging mechanism, complement activation has to be avoided or at least to be restricted on autologous cells (see Table 3 and Figs. 5 and 10). The control efforts are not evenly distributed throughout the activation cascade, but are rather focused on the key events of the pathways leading to C3 activation and on polymerization of C9 (generation of the MAC). There are proteins controlling activation in the fluid phase, i.e., plasma and other membrane-localized proteins that are only effective on the surfaces of autologous or allogeneic cells, but not on xenogeneic cells.

For the classical pathway, activated C1 is soon inactivated by covalent binding of C1-INH to active C1r and C1s. In this complex,

TABLE 3. *Mode of action of complement control proteins*

Control protein	Main site of action	Mode of action			
		Binds covalently to active C1s and C1r			
		Decay acceleration of convertases[a]		Cofactor activity[b]	
		C3b,Bb	C4b,2a	C3b	C4b
C4 activation					
C1-INH	Plasma				
C3 and C5 activation					
Factor H	Plasma and nonactivator membranes	+	−	+	−
C4bp	Plasma	−	+	−	+
CR1	Self[c] membranes (restricted tissue distribution)	+	+	+	+
MCP	Self[c] membranes (wide tissue distribution)	−	−	+	+
DAF	Self[c] membranes (wide tissue distribution)	+	+	−	−
Formation of the membrane attack complex					
S protein	Plasma	Binds to soluble C5b-7 and blocks its integration into membranes			
Clusterin	Plasma	Binds to soluble C5b-7 and blocks its integration into membranes			
CD59	Self[c] membranes (wide tissue distribution)	Inhibits binding of C9 and its polymerization			

[a]Decay acceleration is the ability to dissociate the C3 convertases C3b, Bb or C4b,2a.
[b]Cofactor activity for the cleavage of C3b or C4b by factor I.
[c]In this context, "self" stands for "within the same species." Control proteins are mostly inactive for complement of other species.

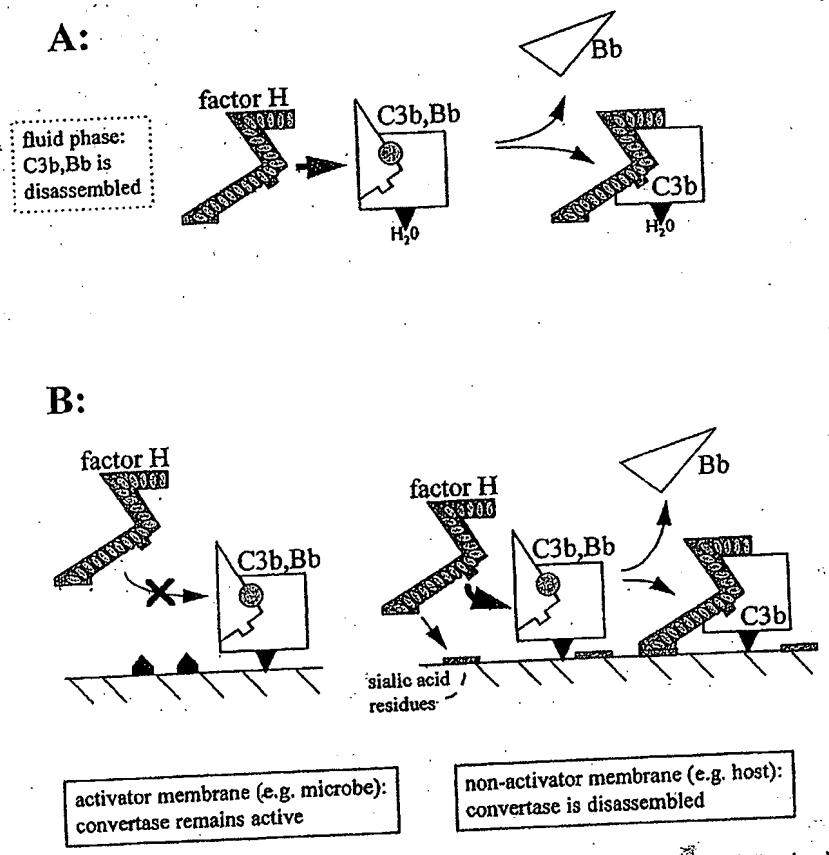

FIG. 10. Control of C3b amplification by factor H. **A:** Factor H destroys C3 convertases circulating in plasma by displacing Bb and rendering C3b accessible for cleavage by factor I. **B:** The same occurs on nonactivator membranes (*right part*) which facilitate factor H binding through sialic acid residues. On activator membranes (*left part*), factor H binding is not promoted and C3b,Bb remains active.

C1s has lost C4 cleaving potential. C1-INH is normally present in a relatively high plasma concentration, and deficiency of C1-INH has been recognized as the cause for hereditary angioedema. C1-INH is probably also involved in the control of MASP-1 and MASP-2.

The next step in control occurs through cleavage of C4b by factor I, which requires C4bp (in fluid phase) or CR1 or MCP (on membranes) as a cofactor. Additionally, the squidlike C4bp binds C4b not attached to a surface, thus preventing association of C4b with C2 in the fluid phase. Control is then exerted on the assembled C3 convertase C4b,2a: the complex is attacked by DAF or CR1 and dissociated into C4b and inactive C2a. This mechanism is equally effective for the C5 convertase of the classical pathway.

The tightest control is afforded for the alternative pathway C3b amplification loop. Convertases present on nonactivator surfaces (Fig. 10B) or in the fluid phase (Fig. 10A) are dissociated into C3b and Bb, which irreversibly deprives Bb of its enzymatic activity, and C3b is cleaved to iC3b, which prevents (re-) formation of a new convertase. First, decay of C3b,Bb is accelerated by the membrane-anchored molecules DAF or CR1 or by the plasma protein factor H. All three are able to displace Bb from C3b, and factor H and CR1 bind to C3b themselves, which is intrinsic for the subsequent cleavage by factor I. Factor H is the main control protein in plasma, but also contributes to dissociation of C3b,Bb on those parts of the cell membrane that are not accessible to DAF (12). Interestingly, DAF uses different SCRs for dissociating C4b,2a or C3b,Bb. It has to be pointed out that without decay accelerators the C3 convertases decay spontaneously, having only a short half-life of 2 minutes. This is important because they are very powerful enzymes and even on activator surfaces C3 activation must be limited.

The inactivation of C3b to iC3b relies on factor I and its cofactors factor H, CR1, or MCP. Due to the high plasma concentration of H, virtually all C3b present in plasma (i.e., nascent C3b that has reacted with water) quickly binds to H. The low value of the Michaelis constant (K_m) of factor I for C3b,H permits an efficient cleavage of C3b (and iC3) even at the low factor I levels in human plasma.

C3b degradation (see Fig. 5) serves two purposes: dangerous C3b is destroyed, but the C3b fragments iC3b and C3dg remain on the activating surface, tagging it for opsonophagocytosis. Factor I cleaves C3b three times: after cleavages 1 and 2, C3b is inactivated to iC3b. The third cleavage releases the larger, biologically inert C3c, whereas the smaller C3dg fragment remains bound to the target as it comprises the thioester region (see Fig. 5). C3dg may be further trimmed by several plasma proteases to C3d.

The physiologic role of the ever-growing family of factor H–like or factor H–related proteins in C3b control is currently not understood (53). Cofactor activity is present in FHL-1, but the low plasma concentration suggests that the protein may have additional characteristics that could be more important (54). FHR-1 and FHR-2 do not have cofactor activity, but share homology in the C terminus of factor H, a site contributing to binding of C3b.

The terminal pathway is controlled both before the integration of the assembling membrane attack complex into the membrane and at the stage of pore formation (association of C8 and polymerization of C9). A number of different membrane and plasma molecules are involved in modulating TCC assembly, of which C8 is probably the most important. It represents not only an essential component of the lytic complex but, paradoxically, also prevents membrane damage by binding to the nascent C5b-7 complex in the fluid phase, thereby precluding its firm insertion into the membrane.

Not only C8, but also the abundant S protein (55), clusterin (also called SP-40,40) (56), lipoproteins, antithrombin III, and proteoglycans such as heparin and protamine, the powerful antidote to heparin, are able to bind to nascent C5b-7 and to prevent its membrane insertion. In addition, numerous interactions have been observed among these inactivators, of which some occur preferably under acidic conditions as reviewed elsewhere (57).

The extent to which these complex interactions affect host defense *in vivo* is not fully understood. The final step of MAC assembly, subsequent to C5b-7 insertion, when the MAC becomes more firmly inserted into the lipid bilayer, is safeguarded by cell membrane proteins, termed homologous restriction factors because they show some degree of species restriction, i.e., they prevent lysis by autologous complement attack (58): (a) a 65- to 68-kDa molecule (C8bp, HRF, MIP), which remains less well characterized and which is supposed to predominantly bind to C8; and (b) an 18- to 20-kDa well-characterized glycolipid-anchored membrane molecule (CD59), which protects against complement-mediated lysis by interfering with the particular C9 interaction site on the C8 α chain that is needed for membrane insertion and subsequent polymerization of C9.

CD59 is found on nucleated cells, including those beyond an (intact) blood–brain barrier (17) and on erythrocytes, but also in serum, urine, seminal plasma, colostrum, and milk. Recently, pigs transgenic for human CD59 have been generated for the envisaged use of xenotransplants in humans, which may be of benefit regarding the shortage of compatible human donors. Such organs have been shown to be protected *in vitro* from hyperacute rejection by expressing human CD59.

COMPLEMENT RECEPTORS

Several biologic activities of complement are mediated by complement receptors that react with activation products generated in the course of one of the activation pathways (see Figs. 4, 11, and 12). Each red and white blood cell expresses cell membrane receptors for various complement fragments (Table 4). It is important to note that native, intact components do not bind to these receptors; the ligands are generated upon activation.

The best studied complement receptors are the cell membrane molecules binding C3 fragments bound covalently to activating surfaces. C3 undergoes degradation that results in cells or particles bearing C3b, iC3b, and C3dg/C3d fragments, forming the ligands for various receptors (Fig. 11). All the receptor binding sites are localized on the α chain of C3. The most important physiologic functions of complement mediated by C3 receptors are the uptake of opsonized particles and activation of various complement receptor–bearing cells.

Complement Receptor Type 1 (CR1, C3b Receptor, CD35)

This single-chain membrane protein binds C3b and C4b with high affinity, and besides serving as a cell membrane receptor, it is involved in the regulation of complement activation (59). CR1 occurs in four polymorphic forms containing up to 34 SCRs. Two of the codominantly expressed allelic forms have MWs of 220 and 250 kDa, and the two other less common forms have MWs of 190

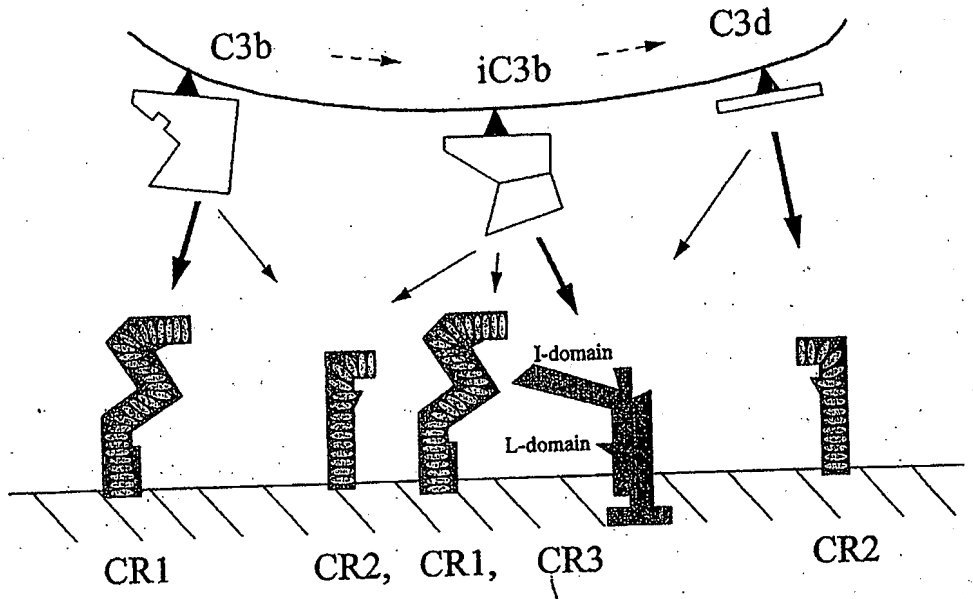

FIG. 11. Specificity of complement receptors for the various fragments of C3. CR1 and CR2 consist of SCRs (shown as ovals), whereas CR3 is a heterodimer (integrin). Higher affinity of a C3 fragment to a certain CR is shown by thicker arrows.

and 280 kDa. The extracellular part of the most common form of CR1 is composed of 30 tandemly arranged SCR domains. These are organized into four groups called long homologous repeats with seven repeated SCRs each (SCRs 1 to 7, 8 to 14, 15 to 21, and 22 to 28) plus two additional SCRs at the C terminus. The ligand binding sites are located on the second SCR in the first three LHRs, providing the basis for multivalent interaction with C3b- or C4b-coated cells and particles.

CR1 is present on erythrocytes, monocytes/macrophages, eosinophils, neutrophils, follicular dendritic cells, and T- and B-lymphocytes. The number of CR1 on erythrocytes is only about 500 per cell, in contrast to leukocytes, where up to 50,000 CR1 per cell can be found. Nevertheless, more than 85% of CR1 in blood is present on the red blood cells because of the vast number of erythrocytes.

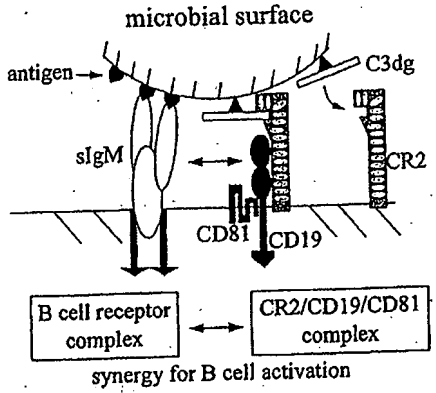

FIG. 12. Interaction of the B-cell receptor complex and the CR2–CD19–CD81 complex for B-cell activation. Antigens coated with C3d bind to the specific surface IgM and to CR2 and cross-link the two receptor complexes. The additional signal triggered via CR2 augments stimulation of the B cell about 100-fold, depending on the amount of C3d bound to the antigen.

The Functions of CR1

The phenomenon of immune adherence, i.e., the binding of opsonized microbes to primate erythrocytes, was the first recognized complement-mediated cellular reaction. This reaction is mediated by CR1 expressed on erythrocytes, a process important for the clearance of immune complexes from the circulation. Soluble antigen–antibody complexes such as toxin–antitoxin complexes are formed after most antibody reactions. These activate the complement system, and C3b that is generated binds covalently to the immune complexes. CR1-expressing erythrocytes adsorb these complexes and transport them to the phagocytic cells of the liver and the spleen for removal.

CR1 expressed on macrophages and polymorphonuclear cells serves as an opsonin receptor. Most probably one of the major defense mechanisms against systemic bacterial and fungal infections is C3b- and iC3b-dependent phagocytosis. On unactivated phagocytes, CR1 alone cannot mediate phagocytosis but efficiently cooperates with Fc receptors and CR3 to bind and ingest opsonized particles. The T cell–derived cytokine IFN-γ and the anaphylatoxic peptide C5a, however, are able to activate macrophages to ingest microbes coated with C3b/iC3b via CR1 only. Triggering of monocytes via their CR1 has been reported to lead to phosphorylation of the receptor and to induce the nuclear translocation of the NF-κB complex (60). As mentioned previously, CR1 also regulates complement activation by the inhibition of C3 convertase activity, thus protecting host cells from comple-

TABLE 4. *Complement receptors*

Type	Ligand	Structure, MW	Distribution	Function
CR1 (CD35)	C3b>C4b>iC3b	Single chain, 160–250 kDa, glycoprotein, four allotypes, consists of 28–34 SCRs	Monocytes, macrophages, neutrophils, eosinophils, erythrocytes, B and T cells, FDC	Immune adherence, phagocytosis, immune complex clearance, immune complex localization to germinal centers, control of activation
CR2 (CD21)	C3db/C3d>iC3b EBV, CD23, IFNα	Single chain, 140–145 kDa, glycoprotein, two isoforms: CD21S (15 SCRs), CD21L (16 SCRs)	B cells, activated T cells, epithelial cells, FDC (CD21L)	B-cell activation, immune complex localization to germinal centers, rescue of germinal center cells from apoptosis
CR3 (CD11b/CD18)	iC3b, factor X, ICAM-1, fibrinogen, LPS, certain carbohydrates	Heterodimer of glycoproteins. α chain: 165 kDa β chain: 95 kDa	Monocytes, macrophages, neutrophils, NK cells, FDC, T cells, mast cells	Phagocytosis, cell adhesion, signal transduction, oxydative burst
CR4 (CD11c/CD18)	iC3b, fibrinogen	Heterodimer of glycoproteins. α chain: 150 kDa β chain: 95 kDa	Monocytes, macrophages, neutrophils, NK cells, T cells, mast cells	Phagocytosis, cell adhesion

ment-mediated damage. Additionally, the genetically engineered soluble form of CR1 also has been shown to inhibit both pathways of complement activation (24). CR1 expressed on follicular dendritic cells in the lymph nodes and spleen plays an important role in maintaining immunologic memory. These cells trap complement-coated immune complexes, enabling the antigen to persist longer in the germinal centers.

Complement Receptor Type 2 (CR2, C3d Receptor, CD21)

Two isoforms of this single-chain glycoprotein have been described: the well-characterized short form of CR2 (CD21S), which comprises 15 SCRs, and the recently reported long CR2 (CD21L), containing an additional exon (encoding an additional SCR 10a). The shorter isoform is expressed on B-lymphocytes, activated T cells, and epithelial cells, but not on monocytes, macrophages, granulocytes, or erythrocytes. The longer CR2 isoform appears to be selectively expressed on follicular dentritic cells (FDC). The ligand binding site of CR2 resides in the first two SCRs. It binds C3dg, C3d, and (weakly) iC3b and also interacts with CD23, the low-affinity Fcε-receptor on B cells. This interaction is thought to be important for isotype switching and survival of germinal center cells (61). However, the highest affinity for CR2 is seen with the envelope protein gp350/220 of Epstein-Barr virus (EBV). Human and mouse CR1 and CR2 proteins are homologous. However, although human CR1 and CR2 are encoded by two separate genes, mouse CR1 and CR2 arise from alternative splicing of a common gene encoding the C3b and C4b binding sites.

The Functions of CR2

Probably the most important physiologic function of CR2 is its recently recognized involvement in B-cell activation by the association with CD19 and TAPA-1 (CD81) in the B-cell membrane (62). Cross-linking of the trimolecular complex to the membrane Ig complex lowers the threshold for B-cell activation and, depending on the number of C3d fragments complexed to the antigen, may enhance Ig production 10- to 10,000-fold (Fig. 12).

Like CR1, CR2 on FDC has been shown to trap immune complexes in germinal centers, most probably playing a role in the development of B-cell memory. The recently described long isoform expressed exclusively on FDC may provide a clue for explaining the mechanism of this process. Pathogenetically very important, CR2 is also the port of entry for EBV, enabling the virus to enter B cells or other CR2-expressing cells. This is achieved without the involvement of complement.

Complement Receptor Type 3 (CR3, Mac-1, CD11b/CD18)

CR3 is a heterodimer containing the 165-kDa α chain (CD11b) and the 95-kDa β chain (CD18) (see Fig. 11). The latter polypeptide is identical with the β chains of LFA-1 and p150,95, the related leukocyte integrins (also named β2 integrins). It is the α chain of the receptor that possesses the binding site for iC3b (and, with lower affinity, for C3b and C3dg). Binding of the ligands to integrins is Ca^{2+} dependent.

The Functions of CR3

CR3 is expressed on mononuclear phagocytes, neutrophils, cytotoxic T cells, FDCs, NK cells, and mast cells. Its most important role is the mediation of binding and phagocytosis of particles and microorganisms opsonized by iC3b. Unlike the interaction between C3b and CR1, binding of iC3b to CR3 is sufficient on its own to initiate phagocytosis. In addition to binding iC3b (via the I-domain), CR3 has carbohydrate-binding capacity (via its lectin or L domain) and in this way interacts with other membrane constituents. Also, some yeasts, such as *Saccharomyces cerevisiae,* and some bacteria, including *Staphylococcus epidermidis,* bind to this receptor without the involvement of complement. Triggering of CR3 via its L domain results in oxydative burst in neutrophils and mononuclear phagocytes. By binding to ICAM-1, CR3 enhances the adhesion of monocytes and neutrophils to the endothelium in the absence of complement proteins and facilitates the accumulation of these cells at sites of tissue injury. Other ligands for CR3 include fibrinogen and clotting factor X. On certain cells, GPI-anchored membrane proteins use CR3 as an adapter for transducing signals across the plasma membrane. The physical and functional association of CR3 with the LPS receptor (CD14) after LPS binding, with the urokinase plasminogen activator receptor (uPAR; CD87) after binding uPA, and with the Fcγ receptor type III (CD16) was demonstrated (63).

Complement Receptor Type 4 (CR4, p150/95, CD11c/CD18)

CR4 is also a heterodimer, containing the 150-kDa α chain and the β chain, which is identical to that of CR3. Both the ligand specificity and the tissue distribution of this receptor is very similar to that of CR3.

RECEPTORS FOR THE ANAPHYLATOXIC PEPTIDES: C5aR (CD88) AND C3aR

In the course of complement activation, peptides of 74 to 77 amino acids are cleaved from the N termini of the α chains of components C4, C3, and C5 (Table 5 and Fig. 13). The cleavage occurs after an arginyl residue, resulting in C4a, C3a, and C5a peptides with C-terminal arginine residues. Receptor molecules for C5a and C3a have been cloned (64). Both C3aR and C5aR are members of the rhodopsin superfamily of receptors, which have seven hydrophobic transmembrane regions and are coupled to G proteins in the cytoplasma (Fig. 13). They are homologous to receptors mediating chemotactic signals, such as the fMLP receptor (which binds bacterial peptides), the receptor for IL-8, or the receptor for RANTES (i.e., chemokine receptor type 5). The deduced MW of C5aR is 43 kDa, whereas C3aR is larger (48 kDa; see Table 5) due to a longer second extracellular loop, most probably conferring ligand specificity.

Functions of C5aR and C3aR

Regarding several of the known biologic activities, C5a is the most potent of all the small activation products, followed by C3a and C4a. Binding of these peptides to their corresponding recep-

TABLE 5. *Receptors for anaphylatoxins and receptors for C1q or factor H*

Type	Ligand	Structure, MW	Distribution	Function
C3aR	C3a	Single chain, 48 kDa, G-protein linked, contains seven transmembrane segments	Mast cells, basophils, smooth muscle cells, lymphocytes	Increases vascular permeability, triggers serosal type mast cells
C5aR (CD 88)	C5a, C5a desArg	43 kDa, single chain, G-protein linked, contains seven transmembrane segments	Mast cells, basophils, neutrophils, monocytes, macrophages, endothelial cells, smooth muscle cells, lymphocytes	Increases vascular permeability, triggers serosal type mast cells, promotes chemotaxis
cC1qR, "collectin-receptor"	Collagen region of C1q, "collectins": MBL, CL-43, SP-A, conglutinin	Single chain, 60 kDa, acidic glycoprotein; identical to endoplasmic reticulum protein calreticulin?	B cell, monocytes, macrophages, platelets, endothelial cells, fibroblasts	
gC1qR	Globular heads of C1q, thrombin, heparin binding form of S protein, Hageman factor, high molecular weight kininogen	33 kDa, acidic protein, tetramer under nondissociating conditions; probably not a surface receptor, but with mitochondrial protein	B cells, monocytes, macrophages platelets, endothelial cells, neutrophils	
$C1qR_p$	Collagenous regions of C1q, MBL, SP-A	Single chain 126 kDa membrane protein, highly glycosylated	Monocytes, macrophages, neutrophils, endothelial cells, microglia	Phagocytosis
fH-R	Factor H	Two species: 150 kDa with 50-kDa subunits 170 kDa, single chain	B cells and B-cell lines, monocytes, macrophages, neutrophils	Activation of B cells, stimulation of respiratory burst, release of factor I, prostaglandin E and thromboxane

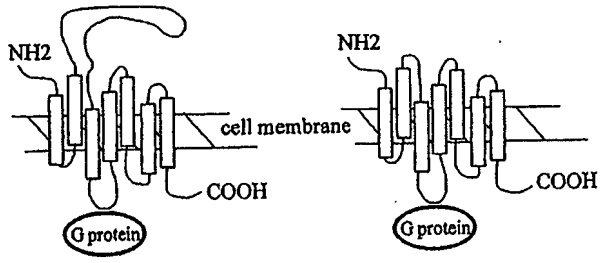

FIG. 13. Structure of the receptors for the anaphylatoxins C3a and C5a. Both belong to the family of seven-pass transmembrane receptors. They signal by interaction of their second cytoplasmic loop with intracellular guanosine triphosphate–binding proteins.

tors induces local inflammatory reactions; therefore, they are often referred to as anaphylatoxins. They induce the contraction of smooth muscle and increase vascular permeability. Consequently, antibodies, complement, and phagocytes are recruited to the site of infection, and a locally developing edema restricts the movement of phagocytes. All these processes contribute to the initiation of adaptive immune responses.

Both C3a and C5a trigger the degranulation of serosal type mast cells, resulting in the release of histamine and other vasoactive mediators from these cells. C3aR and C5aR are widely expressed on different lymphoid cells, and their messenger RNAs have been detected in various nonlymphoid organ tissues. Various cell types respond differently to the anaphylatoxic peptides. On neutrophils, C5a has a strong chemotactic activity and induces the expression of adhesion molecules. These cells are also triggered by the complement peptides to produce oxygen free radicals, prostaglandins, and eicosanoids.

C5a has a short half-life in circulation because the plasma enzyme carboxypeptidase N (also called anaphylatoxin inactivator) cleaves off its C-terminal arginine. C5a desArg generated this way is much less active in several biologic systems than is C5a. In analogy with C5a, serum carboxypeptidase N generates desArg peptides with greatly reduced biologic activity from C3a and C4a.

C1q RECEPTORS

A cell membrane protein named $C1qR_p$, reacting with the collagenlike stalks of C1q, has been cloned recently. This highly glycosylated 126-kDa protein is expressed on phagocytic cells, but not on T- or B-lymphoblastoid cells. It has a C-type carbohydrate recognition domain and five EGF-like domains.

There are two other types of surface proteins that bind to specific regions of the complement subcomponent C1q, although their location at the plasma membrane, and thus their potential to serve as receptors, has recently been questioned (65). The 60-kDa glycoprotein that binds the collagenlike portion of C1q is named cC1qR. This protein is also referred to as the collectin receptor because in addition to C1q, it binds to other members of the collectin family, such as MBL, conglutinin, SP-A and CL-43 (see Table 5). Several cell types, including monocytes, macrophages, B cells, granulocytes, endothelial cells and platelets possess this receptor. Its peptide sequence shows almost complete identity to the complementary DNA–derived sequence of calreticulin, a Ca^{2+} binding protein resident in the endoplasmic reticulum. The relationship between cC1qR and calreticulin is not fully understood. The other acidic cellular protein reacts with the globular heads of C1q and hence has been named gC1qR (see Table 5). The distribution of this 33-kDa protein is very similar to that of cC1qR.

Functions of C1q Receptors

C1q receptors have been reported to mediate several responses of various cells, such as the augmentation of the uptake of bacteria opsonized with MBL and immune complexes having C1q, regulating phagocytosis, eliciting the production of oxygen radicals, and enhancing cell-mediated cytotoxicity.

FACTOR H RECEPTOR

A receptor for factor H has been detected on B-lymphoblastoid cells, monocytes, and neutrophils. Using factor H–sepharose for the isolation of the receptor, a protein complex was identified consisting of two disulfide-linked components of 50 kDa each with an additional 50-kDa chain attached noncovalently. Another cell membrane factor H–binding protein was also isolated from tonsil B cells and from B-lymphoblasts by affinity chromatography. This single-chain protein species was found to be 170 kDa in MW.

Functions of Factor H Receptor

Regarding the possible function of factor H receptor, it has been shown that factor H serves as a growth factor for B-cell lines. Moreover, it stimulates B-lymphocytes and lymphoblastoid cell lines to release endogenous factor I. Factor H also has been demonstrated to trigger the oxydative metabolism of monocytes.

THE ROLE OF COMPLEMENT IN LINKING INNATE IMMUNITY TO ADAPTIVE RESPONSES

Elements of the innate immunity such as the complement system, macrophages, NK cells, and granulocytes are the first-line defense in higher vertebrates. These cells and molecules are able to recognize foreign material and come into action within minutes (or hours) of infection. Several microbes trigger the complement cascade immediately after entering the body in the absence of antibodies by activating either the alternative or the MBL pathway. Complement and complement receptor–mediated processes link innate immunity to adaptive responses in several ways:

- Complement is involved in the initiation of adaptive immune responses because antibody-independent, complement-mediated opsonization of microbes facilitates uptake and presentation of antigens via complement receptor–bearing antigen-presenting cells.
- Antigen-bound C3d facilitates B-cell activation via cross-linking membrane IgM to the CR2/CD19/TAPA-1 complex.

- CR1 and CR2 expressed on follicular dendritic cells are essential for the formation of memory B cells by localizing immune complexes in the germinal centers.
- Complement activation generates anaphylatoxic peptides at sites of infection and, by recruiting inflammatory cells, contributes to the elimination of the antigen.
- Complement plays an important role in the processing of immune complexes by inhibiting the formation of large immune complexes and by solubilizing complexes that have already been precipitated.
- Complement activation resulting in lysis of cells and bacteria is essential for the elimination of several pathogens.

INTERSECTIONS OF THE COMPLEMENT SYSTEM WITH THE CLOTTING AND THE KININ SYSTEM

A biochemical relatedness exists between the complement system and the two other plasma protein systems. For one, the concept for activation in all three relies on consecutive proteolytic cleavages of multiple components, with modified serine protease domains being the effectors. On the other hand, some active components of one system exert effects in one of the others.

For example, C1-INH not only controls C1r and C1s, but also inhibits kallikrein, plasmin, and factors XII and XI of the clotting system (66). Although more potent means of inactivation exist for plasmin or factors XII and XI within the fibrinolytic and clotting system, this is a good example for the overlapping function of a serpin molecule. Plasmin, on its part, is capable of activating C3. Although this is not of importance under physiologic conditions, it is relevant in shock conditions such as disseminated intravascular coagulation or adult respiratory distress syndrome, where concomitant activation of all three systems occurs (67).

COMPLEMENT QUANTITATION

The traditional assay to measure serum complement activity is the total hemolytic complement assay (CH_{50}). In this assay, sheep red blood cells are incubated with an antierythrocyte antibody (amboceptor) and incubated with human serum at various dilutions. The reciprocal of the dilution at which serum lyses 50% of the erythrocytes is the CH_{50} value. This assay measures the functional capacity of only the classical and terminal pathways and is usually combined with immunochemical assays for measuring C3 and C4 protein. These immunochemical assays, which assess the presence and integrity of a protein but not their functional activities, comprise radial immunodiffusion (Mancini), electroimmunodiffusion (rocket electrophoresis, Laurell) and enzyme immunoassay (EIA).

Detecting a reduction in the level of the uncleaved component is less sensitive for assessing complement activation than detecting the increase in cleavage products (C4d, C3dg, C3a, C5a) or complexes containing that particular component (e.g., the TCC). This is readily understood: an increase of a particular concentration from 1% to 5% is much easier to detect (fivefold increase) than a decrease from 99% to 95% (which is within the error of the assay). For these reasons, assays based on activation-specific, so-called neoepitope-specific, and native-restricted monoclonal antibodies have been successfully used to specifically measure only the activated or the native molecule, respectively (68). For accurate assessments they are best run simultaneously because low amounts of native proteins in the first place cannot generate as much activation product as high concentrations. The application of activation-specific antibodies markedly improved both specificity and sensitivity of complement activation assessment in biologic fluids and is used to follow the course of a disease, to reveal exacerbations, and to evaluate the success of a treatment. In particular, these novel methods have been used to assess the biocompatibility of extracorporeal membranes or to distinguish complete from subtotal complement deficiencies. For the latter, the TCC EIA, based on a neoepitope-specific anti-C9 monoclonal antibody, has an additional advantage because it can serve as a functional assay: TCC can only be generated when all preceding proteins, including the one present in limited amounts, are functionally active. However, even these sophisticated assays do not allow, although widely practiced, the assumption that approximately half normal concentrations indicate heterozygous deficiency; even heterozygous subjects may present with almost normal concentrations of the component in question.

COMPLEMENT GENETICS

The study on genetics of complement proteins was originally initiated by the discovery of complement deficiencies in animals and humans. It has been used to detect both homozygous deficient individuals and heterozygous carriers in family studies and to compile further evidence for disease associations with certain complement alleles. However, complement genetics also has been a valuable tool to investigate plasma protein genetics in general and their evolution. The chromosomal assignment of the genes coding for complement proteins (Fig. 14) shows interesting linkage groups of structurally homologous components, confirming previous assumptions, based on homology studies on the protein level, that the majority of complement proteins has evolved by duplication from only a small number of precursor genes (69).

Because complement receptors and certain regulatory proteins are expressed on erythrocytes, they have the potential to represent blood group antigens: the Knops, McCoy, Swain-Langley, and York antigens are known to be on CR1. Variations in the DAF antigen are responsible for the Cromer blood group system, with the rare Inab phenotype lacking DAF altogether. Chido and Rogers blood group antigens are associated with C4 (69). In this respect, complement genetics has been widely applied to anthropologic investigations and forensic medicine.

Recently, progress on the molecular level has facilitated the characterization of complement allotypes on the molecular level. Both phenotypical assessments of protein variants (phenotyping) and characterization of genomic DNA (genotyping) are currently used (70). Phenotyping is traditionally performed using methods analyzing the mobility or isoelectric point of proteins in agarose or polyacrylamide gel electrophoresis. In addition, monoclonal antibodies have been described that distinguish between certain complement allotypes. Genotyping is performed by studying restriction fragment length polymorphisms or by polymerase chain reaction using specific primers followed by enzymatic digestion or sequencing. Phenotyping has the advantage that the presence and, depending on

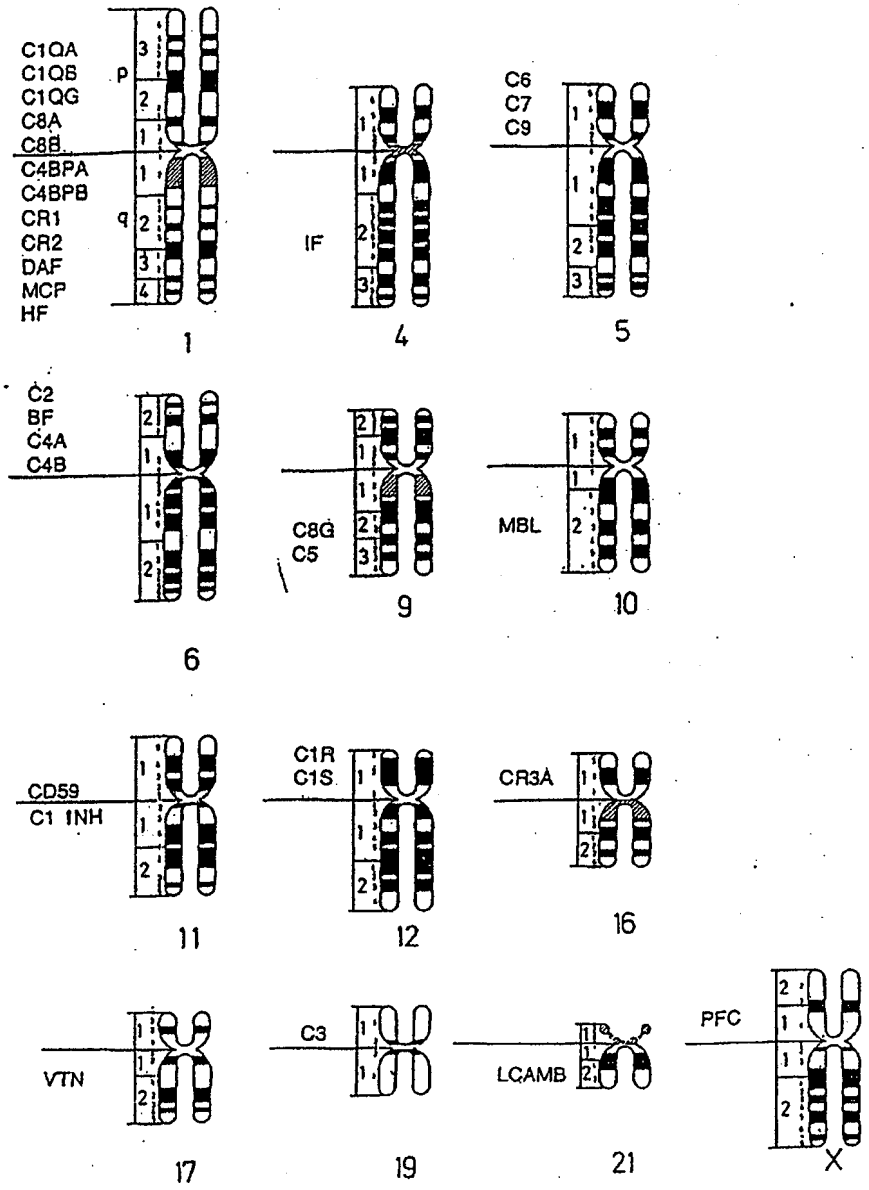

FIG. 14. Schematic diagram of the location of structural genes of complement or related proteins within the human chromosome set, indicated on the left (according to the Paris Nomenclature, Paris Conference, 1971). Only chromosomes carrying complement genes are shown. C1QA, C1q α chain; C1QB, C1q β chain; C1QG, C1q γ chain, all at 1p34-36; C8A, C8 α chain; C8B, C8 β chain, both at 1p22; C4BPA, C4 binding protein α chain; C4BPB, C4 binding protein β chain; CR1, CR2, DAF, MCP, and HF, factor H, all at 1q32 (RCA gene cluster); IF, factor I at 4q25; C6, C7, and C9 at 5p14-p12 (MAC gene cluster); BF, factor B at 6p21 within the MHC III cluster together with C2, C4A, and C4B; C8G, C8 γ chain at 9q22-32 with C5 at 9q33; MBL at 10q22; CD59 at 11p13; C1-INH at 11q12-13; C1R and C1S at 12p13; CR3A, CR3 α chain (CD11b) at 16p13-11; VTN, vitronectin at 17q11; C3 at 19p13; LCAMB, leucocyte adhesion molecule β chain (CD18), common for all β2 integrins, including CR3 at 21q22; PFC, properdin at Xp11.

the method applied, even the functional activity of a protein coded by the allele can be ascertained. Genotyping does not allow identification of silent or null alleles as such; however, once a mutation is known, a defective gene may be traced in family studies, providing a basis for genetic counseling for the afflicted family.

COMPLEMENT AS PATHOGENIC FACTOR IN DISEASE

The complement system contributes to inflammation and tissue damage in neurodegenerative and autoimmune diseases, especially at renal and dermatologic manifestations but also in ischemic and reperfusion injury or shock situations. Evidence has included the detection of complement activation products in biologic fluids or tissues and information from animal models of disease where complement can be efficiently inhibited. Table 6 presents an incomplete summary of these many conditions. In almost all of these, complement is not the cause but is one of several factors involved in pathogenesis (71). In particular, complement is critical to proper immune complex processing. When excessive quantities of complexes are deposited in tissue, ongoing complement activation will also affect and destroy surrounding tissue, such as vascular endothelial cells, leading to vasculitis.

There are probably two ways in which complement fixation influences the fate of immune complexes (72). First, the fixation of C4 and C3 into the antigen–antibody lattice alters the size of the immune complex, giving rise to a large number of small complexes as opposed to a small number of large ones. The latter may precipitate locally and cause Arthus reactions or immune complex disease. Thus, complement helps to solubilize initial immune complexes (detergentlike effect of complement). Second, and probably more important, the presence of C4b and C3b on the immune complex facilitates transport predominantly via the CR1 on red cells in circulation. Under physiologic conditions, erythrocyte-bound immune complexes are sequestered in the liver, where antigenic material can be removed by reticulohistiocytic cells (Fig. 15). If adequate complement fixation on these complexes fails, they can be taken up by endothelial cells and sequestered at peripheral sites, giving rise to further inflammation and immune complex formation.

TABLE 6. *Complement in disease*

	Evidence		
System/Disease	Assay[a]	Histology[b]	Model[c]
Biocompatibility/shock			
Postbypass syndrome	Yes	Yes	Yes
Catheter reactions	Yes	Yes	No
ARDS	Yes	Yes	Yes
Anaphylaxis	Yes	No	No
Transplant rejection	Yes	Yes	Yes
Preeclampsia	Yes	Yes	No
Dermatological			
Pemphigus/pemphigoid	No	Yes	No
Phototoxic reactions	Yes	Yes	Yes
Vasculitis	Yes	Yes	No
Neurological			
Myasthenia gravis	Yes	Yes	Yes
Multiple sclerosis	Yes	Yes	Yes
Cerebral lupus	Yes	No	No
Guillain-Barré syndrome	Yes	Yes	Yes
Alzheimer's disease	No	Yes	No
Renal			
Lupus nephritis	Yes	Yes	Yes
Membranproliferative GN	Yes	Yes	Yes
Membranous nephritis	Yes	Yes	Yes
Rheumatological			
Rheumatoid arthritis	Yes	Yes	Yes
SLE	Yes	Yes	Yes
Behcet's syndrome	Yes	Yes	No
Juvenile rheumatoid	Yes	No	No
Sjogren's syndrome	Yes	No	No
Other			
Atheroma	Yes	Yes	No
Bowel inflammation	Yes	Yes	No
Thyroiditis	Yes	Yes	Yes
Infertility	Yes	Yes	No

[a]Measurement of complement activation products in biological fluids.
[b]Detection of complement products in diseased tissue.
[c]Animal models of disease. Modified (71) with permission.

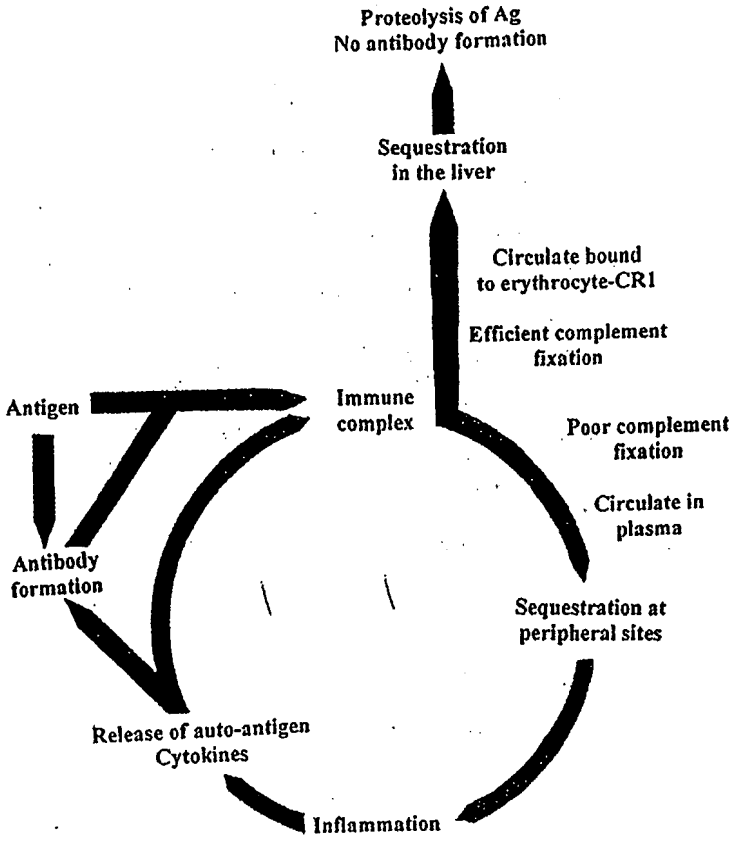

FIG. 15. Schema to show the role of complement in immune complex handling and of defective complement function in giving rise to autoimmune immune complex disease. Reprinted with permission from Oxford University Press (72).

COMPLEMENT DEFICIENCIES

Inherited deficiencies have been described for most complement components and regulatory proteins (Table 7). These abnormalities are relatively rare and usually inherited in an autosomal-recessive manner because only homozygous subjects are readily detected and susceptible to disease. An important exception is hereditary angioedema, which is inherited as an autosomal-dominant trait and also presents in the heterozygote. Complement deficiencies may be considered as important *in vivo* experiments of nature, defining the role of the particular components in the immune system, giving insights into their normal function. Two mutated alleles of the particular gene are usually responsible for the deficiency.

A particular deficiency is paroxysmal nocturnal hemoglobinuria (PNH), which is primarily not a complement deficiency. Mutations in the PIG-A gene affect the synthesis of a competent GPI anchor, which leads to failure of expression of all molecules attached to the membrane via this anchor, including CD55 and CD59. The lack of these two complement control proteins is responsible for the extreme susceptibility of PNH erythrocytes to lysis by complement, activated either by the alternative pathway or via acidic generation of C5-C6 complexes, especially at the physiologically lower blood pH in the night.

The by far most acute, and if untreated potentially lethal, complement deficiency is hereditary angioedema (HAE) due to dysfunctional or missing C1-INH. This disease is intermittently recurring when the patient experiences trauma or infection that forms a trigger for complement activation. Because there is no back-up for C1-INH, activation of C1 will indifferently proceed with generation of C4 and C2 and activation of kinins. The latter are primarily responsible for the classical symptoms of HAE: abdominal colics and suffocation due to larynx edema.

The incidence of complement deficiency states has been difficult to ascertain. A large and representative number of individuals need to be screened, and data available now suggest that the incidence varies considerably depending on the ethnic and geographic background for each component. Study of the relatives of complement-deficient patients and population screening also have led to the identification of a relatively large number (up to 10% to 20%, depending on the component) of healthy deficient individuals.

However, usually complement-deficient subjects are detected because of their increased propensity to infection or in association with immune complex diseases, such as systemic lupus erythematosus (SLE) (Table 7). Particularly striking is the association between SLE and deficiencies of the early classical pathway components. Because only C4 and C2 are coded on the same chromosome (on chromosome 6 within the MHC III gene locus), the possibility that these deficiencies are all linked to a disease susceptibility gene has to be excluded, and there is no reason to question that the increased incidence of these (auto)immune complex diseases is a direct consequence of complement deficiency. Thus, early classical pathway deficiency can be regarded as one of the very few, if not the only, examples where a single defect is sufficient (however, not necessary) for the development of an autoimmune disease (72).

TABLE 7. *Complement deficiency states*

Component	No. of reported patients	Functional defect	Disease associations[a]
C1	50–100	Impaired immune complex handling	SLE, bacterial infections
C4	20–50	Impaired immune complex handling	SLE, bacterial infections
C2	>100	Impaired immune complex handling	SLE, bacterial infections
C3	20–50	Impaired opsonization	Bacterial infections
C1-INH	>>100	Excessive C2 and kinin activation	HAE
B	None		Incompatible with life?
D	3	Impaired alternative pathway activation	Bacterial infections?
P	50–100	Impaired alternative pathway activation	Meningococcal infections
H	<20	Excessive alternative pathway activation	Meningococcal infections, glomerulonephritis
H	20–50	Excessive alternative pathway activation	Bacterial infections
C5	20–50	Impaired chemotaxis, absent lytic activity	Meningococcal infections
C6	>100	Absent lytic activity	Meningococcal infections
C7	>100	Absent lytic activity	Meningococcal infections
C8	>100	Absent lytic activity	Meningococcal infections
C9	>100	Impaired lytic activity	Meningococcal infections

Only strong and established associations are listed, i.e., more than 50% of the diseased subjects have this disease. Note, however, that healthy complement deficient subjects have been found by family studies.

In individuals with homozygous C3 deficiency, pyogenic infections with encapsulated bacteria are severe, recurrent, and life threatening, usually in early childhood. Deficiencies of either factor I or factor H are associated with the inability to degrade C3b, leading to uncontrolled amplification of cleavage of C3 by an unregulated C3b,Bb C3 convertase and result in a state of acquired, severe C3 deficiency (73). Interestingly, the disease associations are not uniform because factor H deficiency, in contrast to C3 or factor I deficiency, predisposes also to glomerulonephritis, which is supported by studies on pig factor H deficiency (74). Deficiency in the factor H–related protein FHR-1 is commonly encountered but has not been linked to any disease (75).

Hereditary deficiency of a terminal complement component leads to an inability to generate a functional terminal complement complex with consecutive absence of hemolysis and bactericidal activity. The particularly frequent occurrence of terminal complement deficiencies in patients with meningococcal infections suggests that the cytolytic activity of the complement system is important in resistance to *Neisseria meningitidis* (76). The data available suggest that either recurrent infection or infection with uncommon serogroups should alert the clinician in Western countries, whereas recurrent disease is the important indicator in endemic areas (77). In addition, it is a quite striking feature that, although *Neisseriae* or Gram-negative bacteria in general have been accused to cause disease in terminal complement deficient subjects, the incidence of gonococcal infections is not increased in deficient subjects, possibly because infections by gonococci are initially restricted to the local mucous membrane and are usually not as fulminantly penetrating into circulation and brain as menongococci. Association of terminal complement deficiencies with susceptibility to autoimmune diseases or nonneisserial infections has been previously mentioned. It was proposed that deficiency might slow down the clearance of these organisms, allowing them to persist for long enough to evoke an abnormal immune response and hence disease (73). Nevertheless, a close examination of the cases available suggests that these associations are very unlikely and probably the result of ascertainment artefacts (77). For example, SLE is found among homozygous terminal complement–deficient subjects, but the frequency is very low and not significantly higher than that found for complement-competent patients.

Several features of terminal complement deficiency have been accumulated in recent years:

1. Low amounts (subtotal deficiency) of functionally active terminal complement proteins may be sufficient for preventing meningococcal disease, suggesting that there is a wide safety margin.
2. Although the incidence of meningococcal infection is much higher, the case fatality rate and the percentage of fulminant cases appears to be lower in terminal complement–deficient subjects when compared with normal subjects. A failure to generate the membrane attack complex with the consequent inability to lyse foreign and autologous cells may lead to a milder form of disease with lower endotoxin concentrations and less host cell injury. In addition, fewer organisms are required for systemic infection. However, in many families of patients investigated, there are often unaccounted deaths of siblings in early childhood, and the possibility of ascertainment artefacts cannot be excluded.
3. The mean age of the first meningococcal attack in complement-deficient individuals tends to be higher than in complement sufficient patients, and the percentage of deficient subjects among meningococcal patients is highest in areas where *N. meningitidis* infections are rare. This reveals that terminal complement deficiency is less likely to be detected in situations where meningococcal infection is common (in early childhood and in meningitis belt countries such as the Sahel zone) and shows that TCC is only one of the means to successfully tackle meningococci.

COMPLEMENT DEFENSE AGAINST INFECTION

Evasion Strategies and Escape of Microorganisms

Microorganisms invading the human body are usually classified by the immune system as nonself. Nonself structures are attacked first by alternative and MBLectin pathways (triggered by the surface composition of the invader) and second by the classical pathway (triggered by specific antibodies targeted toward the intruder,

or directly as in the case of several viruses). Chemotaxis of phagocytic cells, opsonization, and lysis of the microbe then mostly lead to limitation of the attack and control of the infection. This sort of host defense is executed on a number of bacteria, viruses, or fungi, and here typically plays a crucial role.

However, evolution of both host and microorganisms has also created a commensual relationship between humans and several microbes so that in many cases potentially infectious microorganisms are not attacked and live in symbiosis with the host. Most of them only cause disease when the host defense is considerably weakened.

The third type of relationship is medically very important and scientifically the most interesting: microorganisms that are highly pathogenic but nevertheless either evade appropriate recognition or constrain suitable attack and destruction (78). To achieve these goals, a range of strategies has been developed by microorganisms during evolution, including both biochemical and biophysical measures to resist C3b deposition, opsonophagocytosis, or complement-mediated cytolytic damage (Table 8) or the remarkable mimicking of complementlike structures or functions (Table 9). A number of microorganisms even use complement receptors to initiate infection in two ways. More commonly, the microorganisms have refined complement-activating properties, which lead to nonopsonic attachment of C3 fragments on their surface, resulting in an inappropriate recognition by polymorphonuclear cells (PMNs) (disguise) (Table 8). On the surface

TABLE 8. *Resistance to complement-mediated damage by interfering with or using complement, excluding molecular mimicry*

Interference with complement activation via poorly activating molecules on the surface of the pathogen	
Lipopolysaccharide	*Salmonella spp., Klebsiella pneumoniae*
Sialic acids	*Schistosoma mansoni*
Trypsin/sialidase sensitive molecules	*Trypanosoma cruzi*
Serum-resistant promastigotes	*Leishmania spp.*
Interference with complement activation via C1q/C1s binding proteins on the surface of the pathogen	
Inhibiting[a]	*Salmonella minnesota* porin, 39 kDa
Inhibiting	*Schistosoma mansoni* paramyosin
Inhibiting	*Taenia solium* paramyosin
Enhancing[a]	HIV-gp41
Interference with C3 convertases	
Blocking of assembly	*Streptococcus spp., Campylobacter spp.*
Prevention of access of phagocytes to cell surface C3 fragments	
Development of capsules	Several encapsulated bacteria
Adsorption of C3 fragments on the pathogen to gain entry into a target cell (usually monocyte/macrophage)	
Utilization of Cr1, target cell: erythrocyte	*Babesia rodhaini, Plasmodium spp.* merozoites
Utilization of CR1 and CR3	*Legionella pneumophila, Mycobacterium leprae, Mycobacterium tuberculosis, Leishmania major*
Utilization of CR2	HIV-1
Utilization of CR3	HIV-1 West Nile virus
Interference with complement activation after C3 generation	
No deposition of C6 or C9 on membrane	*Borrelia burgdorferi*
Inhibition or inactivation of C5b6	*Escherichia coli* (traT)
C3b binding far from membrane, no MAC assembly	*Klebsiella pneumoniae*
Interference with C5b-9 insertion into the cytoplasmic membrane	
Insertion distant from membrane due to	
Hydrophobic outer membrane constituents	*Salmonella spp.* (rck), *Trypanosoma cruzi*
Restriction of bactericidal process[a]	*Neisseria gonorrhoeae* (porin PI)
?	*Yersinia enterocolitica* (YadA)
?	*Moraxella catarrhalis*
Incorporation into soluble C5b-9, clusterin binding	*Streptococcus pyogenes A* (SIC)
Adsorption, incorporation or expression of complement regulatory proteins	
Attachment of factor H	*Streptococcus A* (M-protein)
Adsorption of DAF	HIV-1
Incorporation of DAF	Cytomegalovirus
Expression of DAF	*Schistosoma mansoni*
Expression of MCP	Cytomegalovirus
Incorporation of CD59	HIV-1
Proteolytic cleavage of complement components	
Cleavage of C1q and C3	*Pseudomonas deruginosa*, elastase and alkaline protease
Cleavage of C1-INH	*Serratia marcescens*, protease, 56 kDa
Cleavage of C3	*Porphyromonas gingivalis*, trypsin-like protease, 80 kDa *Entamoeba histolytica*, cystein protease, 56 kDa *Leishmania major*, acid protease, gp63
Cleavage of C3 and C9	*Schistosoma mansoni*, serine protease, 28 kDa
Cleavage of C4, C3, C5–C9	*Serratia liquefaciens*, metalloprotease, 53 kDa

[a]assumed/proposed.
? not known.

TABLE 9. *Resistance to complement-mediated damage by mimicking complement proteins*

	Ligand	Functional mimicry	Structural similarity	Antigenic cross-reactivity	Sequence homology
Mimicry of C3 Convertase controlling proteins (DAF, C4bp)					
Blocking assembly of CP C3 convertase					
Trypanosoma cruzi gp60	C3b	CR1, DAF			DAF
Blocking assembly of AP C3 convertase					
Trypanosoma cruzi gp160					
Trypanosoma cruzi gp58/68					
Blocking assembly of CP and AP and accelerating decay of CP C3 convertase					
Vaccinia virus VCP, gp35	C4b, C3b	CR1	C4bp		C4bp,DAF,MCP
Accelerating decay of AP C3 convertase					
Herpes simplex virus-1/2 gC 1/2	C3b,iC3b	CR1		CR1[a]	CR1
Epstein-Barr virus	C3b,iC3b,C4	CR1			
Herpes virus Saimiri CCPH (ORF-4)	C4bp	DAF		C4bp,DAF,MCP	
Accelerating decay of CP and AP C3 convertase					
Trypanosoma cruzi gp87–93		CR1, DAF		DAF	
Schistosoma mansoni	C3, C4	CR1			
Unknown mode					
HIV-1 gp120	iC3,C3b,C4b	C4bp		C4bp	C4bp
Mimicry of C3 and C4 facilitating receptor binding					
Epstein-Barr virus gp350/220	CR2	C3d/C3dg		C3d/C3dg	C3d/C3dg
HIV-1 gp41	CR3	C3			C3
Mycobacterium tuberculosis	CR3	iC3b			
Leishmania major/donovani gp63	CR3, CR4	iC3b			
Histoplasma capsulatum	CR3, CR4	iC3b			
Measles virus	MCP				
Streptococcus pyogenes A M-protein	MCP				
Enteroviruses (ECHO, Coxsackie)	DAF				
HIV-1 gp41	P,H	C3			C3
Streptococcus pyogenes A	C4bp	C4b			
Mimicry of thrombospondin repeat containing proteins					
HIV-1 gp120	C3b,H	P		P	P
Plasmodium falciparum TRAP and CSP	CR1[a]		P,C6-C9		
Trypanosoma cruzi hemolysin, 75 kDa	C9			C9	
Mimicry of complement receptors facilitating adhesion					
HIV-1 gp120	C3b	CR3			CR3 (CD11b)
Candida albicans, CR3-like, 188 kDa	iC3b	CR3	CR3 (CD11b)	CR3 (CD11b)	CR3 (CD11b)
Candida albicans, CR2-like	C3b	CR2		CR2	
Mimicry of CD59 limiting membrane attack					
Herpes virus Saimiri ORF-15 (ORF-LS)	C9[a]	CD59	CD59		
Entamoeba histolytica adhesin, 260 kDa	C8, C9	CD59		CD59	CD59
Schistosoma mansoni, SCIP-1	C8, C9	CD59		CD59	

[a]assumed/proposed.
AP alternative pathway; CP, classical pathway

of some microorganisms, however, proteins antigenically or functionally mimicking C3 are present that can bind to complement receptors, mediating uptake in a complement-independent manner, i.e., the uptake does not rely on prior opsonization of the invader (Table 9). By both means, disguise and mimicry, the pathogen avoids destruction by complement and antibody and can harness the cellular machinery for its own reproduction. However, it should be stressed that complement resistance may depend on molecules other than proteins.

An interesting additional feature is the proteolytic degradation of complement proteins by microorganisms protecting them from opsonization or lysis (Table 8). Cleavage of C1-INH by proteases leads to constant consumption of C1 and cleavage of C3, leading to proinflammatory responses and reactive lysis of bystander cells. These microbes must have a highly sophisticated regulation to ensure that enough but not too much detrimental activation occurs. This is also true for pathogens, using a particular receptor for their entry into the host cells. Cleavage has to be very accurate so that most of the surface-deposited C3 is present in the optimum form (C3b or iC3b) for receptor binding.

Another mechanism is the use of complement proteins provided by the host. When HIV-1 is leaving an infected cell (budding process), it is encoated by a lipid bilayer obtained from the host cell membrane and as a consequence carries, in addition to viral, also host cell membrane proteins. Of the latter, DAF and CD59 are of particular importance because they protect HIV-1 from complement lysis (79). Attachment of factor H to C3b on the virus and to several sites on the external portion of gp41 and to one site on gp120 additionally protects against efficient destruction (80,81).

Mimicry of Complement Structures by Microorganisms

During millions of years of coevolution alongside their obligate hosts, several pathogenic microorganisms have evolved functional properties identical to those used by normal mammalian cells to prevent their own destruction by complement. In particular, a number of distinct microbial proteins have been identified that share structural or genetic similarities (antigenic cross-reactivity, sequence homology) with complement proteins or receptors. Such molecular mimicry not only enables the pathogens to avoid destruction by complement, but also facilitates complement-mediated infection via complement receptors (82). Under certain circumstances, mimicry can even lead to development of autoimmunity.

Furthermore, in some instances only a certain principle is adopted. Several microorganisms attack human cells by drilling holes into the lipid bilayer using polymerization and cylinder formation of their specific cytolysins: streptococcal streptolysin-O, *E. coli* hemolysin, or staphylococcal α-toxin (83). The presence of these pore-forming proteins is strongly associated with the virulence of their carriers. However, although using a similar biologic principle as C9, these microbial toxins do not exhibit structural homology on the amino acid sequence level to each other or to C9 or perforin. A number of these molecules bringing about lysis have been identified (84). Some of the microbial proteins mimicking complement proteins are listed in Table 9, comprising mostly those that have yet been defined on the molecular level. For some of these, however, data are insufficient to support their postulated involvement *in vivo* in immune evasion, and many more are awaited to fill this list.

The question is how these molecules have evolved. Teleologically, some of the complementlike molecules are expressed as a consequence of selection on the basis of facilitation of attachment, penetration into host cells, or escape from lysis (84). In the case of vaccinia virus, the DNA encoding VCP, a functionally CR1-like and structurally C4bp-like complement control protein, was presumably originally acquired from the host. Over an evolutionary period, the captured gene was constantly manipulated to retain only the most essential domains because any further manipulation of the small viral protein results in loss of function, indicating that the gene has achieved a maximum efficiency to encode a protein with the minimum number of amino acids (85). In other pathogens, molecular mimicry may represent the conservation of important ancestral molecular recognition motifs. Some of the molecules listed in Table 9 are discussed here in more detail. Many are related to mammalian CR1, DAF, MCP, or C4bp, confirming the importance of C3- and C4-binding molecules.

The overall homology of HIV envelope proteins gp41 and gp120 with complement proteins is very low; in certain short stretches, however, remarkable similitaries were discovered. The respective sites appear to be involved in complement binding and may facilitate virus uptake via complement receptors or play a role in the noncovalent association between gp41 and gp120 (80).

The trematode *Schistosoma mansoni* appears to have the most elaborate anticomplement arsenal: first, it can modify its surface sialic acids, thus modulating activation; second, it can acquire DAF to accelerate decay of surface-bound C3; third, it can bind and cleave C4 and C3 mimicking CR1; fourth, it can cleave C9, preventing MAC assembly; and fifth, but probably not last, it encodes a protein mimicking CD59, inhibiting membrane attack.

The yeast *Candida albicans* possesses an integrin/CR3-like molecule on its surface that is involved in morphology changes representing a virulence factor (86). Furthermore, it appears to facilitate cellular adherence like all members of the human integrin gene family. Interestingly, this molecule is not only functionally (87) but also antigenically and structurally related to human CR3. There is strong evidence that HIV-1 is able to bind to *Candida* directly, possibly via C3-like regions on gp41 and CR3-like regions on *Candida* (88). This interaction enhances candidial proteinase release and suppresses phagocytosis by PMNs (89). Thus, the concerted mimicry of both pathogens may contribute to the virulence of both *Candida* and HIV (88).

It has been proposed that sites of molecular mimicry may represent useful sites for vaccine development (90). However, considering the multiple as yet unrevealed interactions, a detrimental effect of such a vaccine cannot be excluded.

COMPLEMENT DISORDERS AND CLINICAL THERAPY

Effector functions arising from activated complement proteins are potentially harmful with the consequence of inflammatory tissue destruction. This is manifested clinically in various diseases, including sepsis and multiple organ failure. In animals, complement depletion or the use of hereditary deficient species has been effective in reducing tissue injury and ameliorating disease.

Therapeutic interventions to prevent complement activation, to control complement-mediated inflammation, and to minimize host cell lysis are promising and offer tremendous clinical potential for

treating a wide variety of acute and chronic diseases. Attempts to efficiently inhibit complement include the following:

1. The application of endogenous purified complement inhibitors (C1-inhibitor)
2. The use of recombinant soluble complement inhibitors (recombinant soluble CR1)
3. The administration of antibodies blocking key steps in the cascade reaction, such as the formation of TCC assembly or C5a generation (anti-C5)
4. The treatment with neutralizing antibodies that inhibit anaphylatoxin effects on host tissue (inflammation, anti-C5a, anti-C5aR)
5. The use of antibodies interfering with adhesion of inflammatory cells to the vascular endothelium (anti-CR3)
6. The incorporation of membrane-bound complement regulators into organs of transgenic animals (pigs) as xenograft sources (CD55, CD46, and CD59) (91)

SUMMARY AND CONCLUSIONS

When immunologists started to dissect the functional entities of the humoral immune response in the second half of the last century, one of the fundamental observations was the discrimination between heat-stable and heat-labile factors. The heat-stable, antigen-specific component was termed the antibody, whereas the heat-labile factor was thought to assist antibodies of diverse specificities in their destructive work and hence given the name "complement" by Paul Ehrlich. However, the role of an executor in the course of the humoral immune response characterizes only one (i.e., the classical pathway) of three pathways leading to the pivotal step of complement activation, i.e., generation of C3b from C3 and its covalent deposition on the activating surface. Likewise, the common endpiece of all complement activation pathways, the terminal pathway, finally leads to assembly of the pore-forming, lytic membrane-attack complex.

Phylogenetically older than the classical pathway, the alternative pathway forms a primitive immune system on its own. It recognizes microbial surfaces in a way distinct from antibody and directs deposition of C3b to these particles. Host tissue is protected by a powerful, redundant control mechanism from self-destructive alternative pathway activation.

The more recently discovered MBLectin pathway has most of the biochemical steps in common with the classical pathway, but is triggered by binding of MBL to polysaccharides present on the surface of many microbes. The importance of all three pathways for innate and acquired immunity is reflected by genetically caused deficiencies that either relate to increased susceptibility to infection or to immune-complex disease.

The molecular core of complement is formed by two main protein families. First, the thioester proteins C3 and C4 possess the unique ability to attach covalently to surfaces upon activation, which causes a profound conformational change. Second, several plasma and membrane proteins interact with the thioester proteins via SCR modules. The SCR is a structural unit that comprises about 60 amino acids (10 to 16 highly conserved). On the one hand, SCRs are found in complement proteins that advance complement activation (e.g., C1r and C1s, C2, factor B). Additionally, these proteins possess serine protease domains to perform the initial proteolytic steps on C3 and C4 required for the amplification of the activation cascade. On the other hand, SCRs are abundantly present in complement receptors (CR1, CR2) or in complement regulatory proteins (e.g., factor H, DAF), which restrict complement activation by binding to activated forms of C3 or C4 and bringing about their inactivation. The genes of these proteins constitute the RCA gene cluster.

The complement system has been present throughout the evolution of the vertebrates, and primitive forms of it are found among nonvertebrates. Ever since, pathogens have tried to overcome this major obstacle or to use it to their advantage. Molecular mimicry is documented among all sorts of pathogens, from nematodes and protozoa to bacteria and viruses. For example, *Trypanosoma cruzi* disposes of proteins that resemble complement regulators present on the host cell and thus help the parasite to evade complement attack. Other pathogens like *Leishmania* or the Epstein-Barr virus use the host cell's complement receptors as their port of entry.

Although designed to combat microbes, complement activation may become harmful to the host himself under certain circumstances. Excessive complement activation contributes to the pathology of immune-complex diseases or autoimmune syndromes. To interfere with such unwanted complement activation has been a longstanding goal of complement research. Recent achievements like recombinantly produced forms of complement regulator proteins (soluble CR1) or humanized monoclonal antibodies against C5 have met the first expectations for a use in several clinical settings. On the other hand, the range of pathologic disorders, where the role of complement is being scrutinized, is broadening. Besides infectious or rheumatic diseases, it now also encompasses atherosclerosis, Alzheimer's disease, and cancer. Consequently, renewed clinical interest will lead the way into the second century of complement research.

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HUMAN PHYSIOLOGY

The Mechanisms of Body Function

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Exhibit J

HUMAN PHYSIOLOGY:
The Mechanisms of Body Function

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This book was set in News Gothic by Graphic Services, Inc.,
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and the *platelets*. Ordinarily, the constant motion of the blood keeps the cells well dispersed throughout the plasma, but if a sample of blood is allowed to stand (clotting prevented), the cells slowly sink to the bottom. This process can be speeded up by centrifuging. By this means, the percentage of total blood volume which is cells, known as the *hematocrit,* can be determined. The normal hematocrit is approximately 45 percent. The total blood volume of an average man is approximately 8 percent of his total body weight. Accordingly, for a 70 kg man

total blood weight = 0.08 × 70 kg = 5.6 kg

One kilogram of blood occupies approximately 1 liter; therefore

total blood volume = 5.6 liters

The hematocrit is 45 percent; therefore

total cell volume[1] = 2.52 liters
plasma volume = 5.6 − 2.52 liters = 3.08 liters

Plasma is an extremely complex liquid. It consists of a large number of organic and inorganic substances dissolved in water. The most abundant solutes by weight are the proteins, which together compose approximately 7 percent of the total plasma weight. The *plasma proteins* vary greatly in their structure and function, but they can be classified, according to certain physical and chemical reactions, into two broad groups, the *albumins* and the *globulins*. The albumins are three to four times more abundant than the globulins and usually are of smaller molecular weights. The plasma proteins, with one notable exception, are synthesized by the liver, the exception being the group known as *gamma globulins,* which are formed in the lymph nodes and spleen (Chap. 16). The plasma proteins serve a host of important functions which will be described in relevant chapters, but it must be emphasized that normally they are *not* taken up by cells and utilized as metabolic fuel. Accordingly, they must be viewed quite differently from most other organic constituents of plasma, such as glucose, which use the plasma as a vehicle for transport but function in cells. The plasma proteins function in the plasma itself or, under certain circumstances, in the interstitial fluid.

[1]Since the vast majority of all blood cells are erythrocytes, the total cell volume is approximately equal to the erythrocyte volume.

TABLE 10-1 Plasma Concentrations of Electrolytes and Protein

CONSTITUENT	GRAMS PER LITER	MILLIMOLES PER LITER
Sodium, Na^+	3.39	144
Chloride, Cl^-	3.55	100
Bicarbonate, HCO_3^-	1.50	25
Potassium, K^+	0.17	4.4
Calcium, Ca^{++}	0.10	2.5
Phosphate, HPO_4^{--} or $H_2PO_4^-$	0.10	1.0
Magnesium, Mg^{++}	0.04	1.5
Protein	70	2.5

In addition to the organic solutes—proteins, nutrients, and metabolic end products—plasma contains a large variety of mineral electrolytes, the concentrations of which are shown in Table 10-1, along with that of protein. The value in millimoles per liter for protein may seem puzzling in view of the statement that protein is the most abundant plasma solute by *weight*. Remember, however, that molarity is a measure not of the weight but of the *number* of molecules or ions per unit volume. Protein molecules are so large in comparison with sodium ions that a very small number of them greatly outweighs a much larger number of sodium ions. The osmolarity (and, therefore, water concentration) of a solution depends upon the *number,* not the weight, of the solute particles present. Accordingly, sodium is the single most important determinant of total plasma osmolarity.

OVERALL DESIGN OF THE CARDIOVASCULAR SYSTEM

The cardiovascular system (Fig. 10-1) comprises a set of tubes, *blood vessels,* through which blood flows and a pump, the *heart,* which produces this flow. Physiology as an experimental science began in 1628, when William Harvey demonstrated that the entire system forms a circle, so that blood is continuously being pumped out of the heart through one set of vessels and returning to the heart via a different set. In man, as in all mammals, there are actually two circuits, both originating and terminating in the heart, which is divided longitudinally into two functional halves. Blood is pumped via one circuit (the *pulmonary circulation*) from the right half of the heart through the lungs and back to the left half of the heart. It is pumped via the second circuit (the *systemic circulation*) from the left half of the heart

Figure 3.2.S.1.2.2: Expected Amino Acid Sequence for h5G1.1-mAb

Heavy Chain
QVQLVQSGAEVKKPGASVKVSCKASGYIFSNYWIQWVRQAPGQGLEWMGEILPGSGSTEYTENFKDRVTMTRDTSTSTVYMELSSLR SEDTAVYYCARYFFGSSPNWYFDVWGQGTLVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVERKCCVECPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVV VDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVY TLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHY TQKSLSLSLGK N-Terminal = Pyroglutamate C-Terminal = OH Average Mass = 49,502 Da
Light Chain
DIQMTQSPSSLSASVGDRVTITCGASENIYGALNWYQQKPGKAPKLLIYGATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQ NVLNTPLTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC N-Terminal = H C-Terminal = OH Average Mass = 23,135 Da

Exhibit K

ORIGINAL ARTICLE

Effect of Eculizumab on Hemolysis and Transfusion Requirements in Patients with Paroxysmal Nocturnal Hemoglobinuria

Peter Hillmen, M.B., Ph.D., Claire Hall, M.B., Ch.B., Judith C.W. Marsh, M.B., M.D., Modupe Elebute, M.B., M.D., Michael P. Bombara, B.S., Beth E. Petro, B.S., Matthew J. Cullen, B.Sc., Stephen J. Richards, Ph.D., Scott A. Rollins, Ph.D., Christopher F. Mojcik, M.D., Ph.D., and Russell P. Rother, Ph.D.

ABSTRACT

BACKGROUND

Paroxysmal nocturnal hemoglobinuria (PNH) arises from a somatic mutation of the *PIG-A* gene in a hematopoietic stem cell and the subsequent production of blood cells with a deficiency of surface proteins that protect the cells against attack by the complement system. We tested the clinical efficacy of eculizumab, a humanized antibody that inhibits the activation of terminal complement components, in patients with PNH.

METHODS

Eleven transfusion-dependent patients with PNH received infusions of eculizumab (600 mg) every week for four weeks, followed one week later by a 900-mg dose and then by 900 mg every other week through week 12. Clinical and biochemical indicators of hemolysis were measured throughout the trial.

RESULTS

Mean lactate dehydrogenase levels decreased from 3111 IU per liter before treatment to 594 IU per liter during treatment ($P=0.002$). The mean percentage of PNH type III erythrocytes increased from 36.7 percent of the total erythrocyte population to 59.2 percent ($P=0.005$). The mean and median transfusion rates decreased from 2.1 and 1.8 units per patient per month to 0.6 and 0.0 units per patient per month, respectively ($P=0.003$ for the comparison of the median rates). Episodes of hemoglobinuria were reduced by 96 percent ($P<0.001$), and measurements of the quality of life improved significantly.

CONCLUSIONS

Eculizumab is safe and well tolerated in patients with PNH. This antibody against terminal complement protein C5 reduces intravascular hemolysis, hemoglobinuria, and the need for transfusion, with an associated improvement in the quality of life in patients with PNH.

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Exhibit L

THE MAJOR CLINICAL SIGNS OF PAROXysmal nocturnal hemoglobinuria (PNH) are intravascular hemolysis, venous thrombosis, and hemoglobinuria.[1] The disease arises from a somatic mutation of the PIG-A gene in a pluripotent hematopoietic stem cell. PIG-A encodes a protein that is essential for the synthesis of glycosylphosphatidylinositol (GPI), a lipid moiety that is embedded in the plasma membrane, where it serves to anchor a wide variety of proteins to the cell surface. The mutant stem cell subsequently expands to form a hematopoietic clone with a deficiency in proteins that are normally attached to the cell surface by the GPI anchor.[2,3] The mature blood cells derived from the hematopoietic clone can have a complete deficiency (type III) or a partial deficiency (type II) of GPI-linked proteins and almost always coexist with residual cells with a normal expression of these proteins (previously identified as type I).

The clinical features of PNH result from the lack of one or more GPI-linked proteins that protect cells from complement-mediated attack. Two such proteins — CD55 and CD59 — are absent from PNH type III erythrocytes, platelets, and other blood cells.[4-6] CD55 regulates early complement activation by inhibiting C3 convertases,[7] whereas CD59 inhibits the assembly of the membrane-attack complex C5b–C9 by interacting with C8 and C9.[4,5] The lack of CD59 is probably responsible for the increased sensitivity of PNH erythrocytes and platelets to complement.[4,8-13]

Eculizumab is a recombinant humanized monoclonal antibody that was designed to block the activation of terminal complement components.[14,15] It binds specifically to the terminal complement protein C5, inhibiting its cleavage into C5a and C5b, thereby preventing the release of the inflammatory mediator C5a and the formation of the cytolytic-pore C5b–C9. Blockade of the complement cascade at C5 preserves the early components of complement that are essential for the opsonization of microorganisms and clearance of immune complexes.[16] In this trial, we investigated whether eculizumab could reduce the incidence of intravascular hemolysis, hemoglobinuria, and transfusion requirements in patients with PNH.

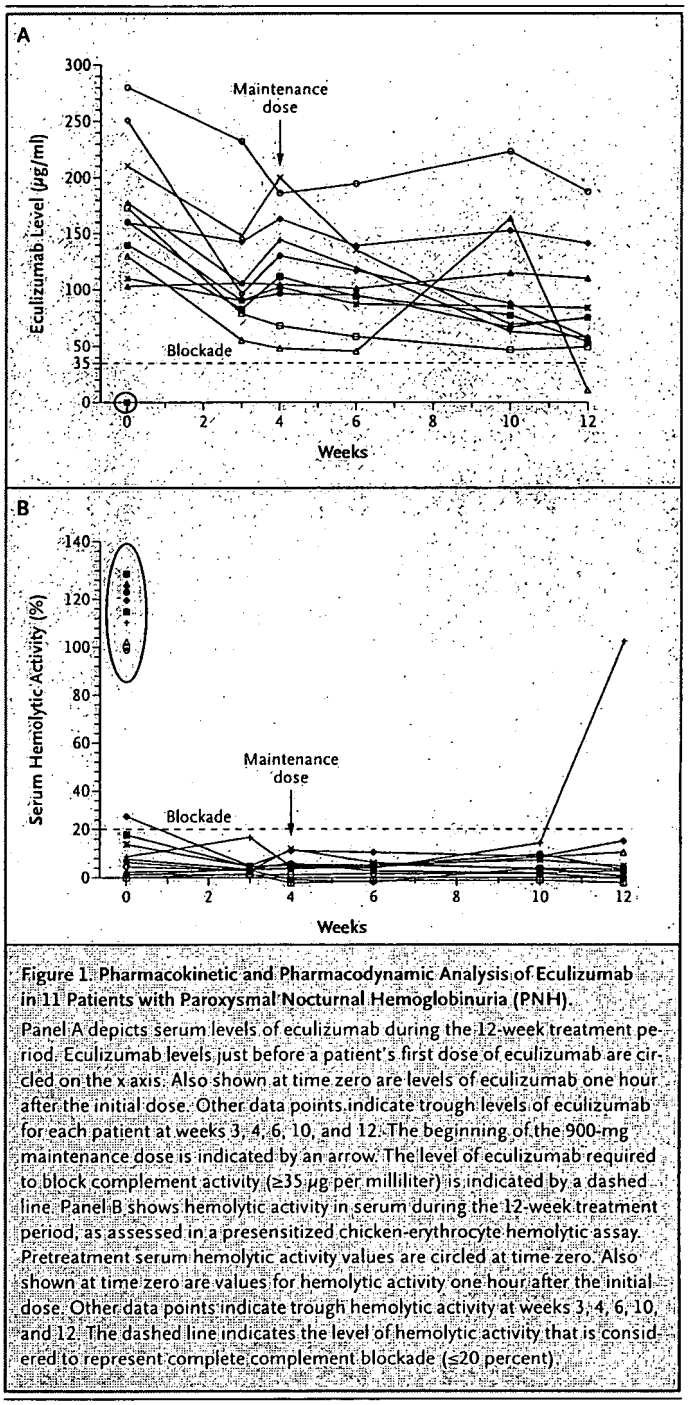

Figure 1. Pharmacokinetic and Pharmacodynamic Analysis of Eculizumab in 11 Patients with Paroxysmal Nocturnal Hemoglobinuria (PNH).

Panel A depicts serum levels of eculizumab during the 12-week treatment period. Eculizumab levels just before a patient's first dose of eculizumab are circled on the x axis. Also shown at time zero are levels of eculizumab one hour after the initial dose. Other data points indicate trough levels of eculizumab for each patient at weeks 3, 4, 6, 10, and 12. The beginning of the 900-mg maintenance dose is indicated by an arrow. The level of eculizumab required to block complement activity (≥35 μg per milliliter) is indicated by a dashed line. Panel B shows hemolytic activity in serum during the 12-week treatment period, as assessed in a presensitized chicken-erythrocyte hemolytic assay. Pretreatment serum hemolytic activity values are circled at time zero. Also shown at time zero are values for hemolytic activity one hour after the initial dose. Other data points indicate trough hemolytic activity at weeks 3, 4, 6, 10, and 12. The dashed line indicates the level of hemolytic activity that is considered to represent complete complement blockade (≤20 percent).

METHODS

PATIENTS

The study was conducted from May through December 2002. Men and women (18 years of age and older) who had received a diagnosis of PNH at least six months earlier, had a detectable GPI-deficient hematopoietic clone, and had received at least four red-cell transfusions in the preceding 12 months were

eligible. Patients were required to have a negative throat culture for *Neisseria meningitidis* and *N. gonorrhoeae*. All patients were vaccinated against *N. meningitidis* (Mengivac (A+C), Aventis Pasteur) before treatment. One patient had a stroke after consent but never received eculizumab and was excluded. Patients who were taking stable doses of immunosuppressive drugs (e.g., cyclosporine), warfarin, and iron supplements were permitted to continue them.

The trial was approved by the local research ethics committee and was performed according to the International Conference on Harmonisation and Good Clinical Practice Standards. Eleven patients gave written informed consent and were treated with eculizumab.

TREATMENT SCHEDULE

Patients received infusions of 600 mg of eculizumab weekly for four weeks, followed one week later by a 900-mg dose and then by a dose of 900 mg every other week through week 12.

INVESTIGATIONS

In this open-label pilot study, we obtained data on the pharmacokinetics, pharmacodynamics, and immunogenicity of eculizumab and observed its clinical effects by measuring the following: lactate dehydrogenase, haptoglobin, bilirubin, and hemoglobin levels; reticulocyte counts; the proportion of GPI-deficient cells, as assessed by flow cytometry[17]; the rate of transfusion with packed red cells; the rate of occurrence of hemoglobinuria (assessed by daily comparison of the first morning urine sample with a standardized color chart before and during treatment); and the quality of life, as reflected by the scores on the European Organization for Research and Treatment of Cancer (EORTC) QLQ-C30 instrument. The trigger for transfusion during the study period remained unchanged for each patient, as compared with their care before entry into the study: patients received blood transfusions when they had symptoms resulting from anemia.

Assessment of the safety of eculizumab included ascertainment of treatment-related adverse events, electrocardiography, and routine laboratory tests (e.g., serum chemical analyses and complete blood counts).

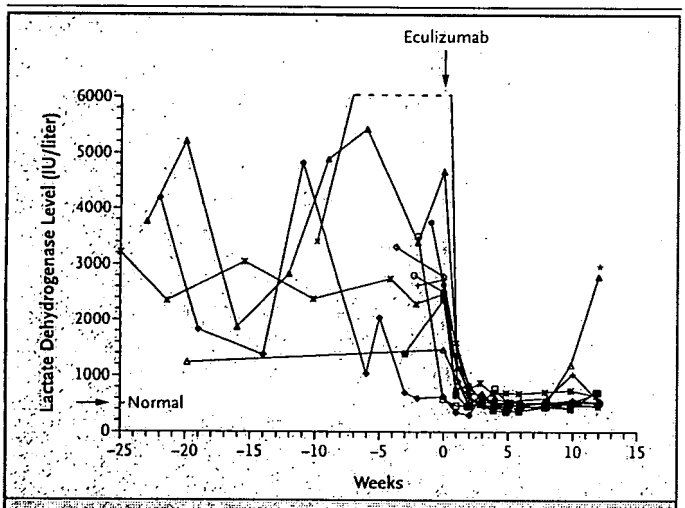

Figure 2. Analysis of Lactate Dehydrogenase Levels, a Biochemical Indicator of Hemolysis, in 11 Patients with Paroxysmal Nocturnal Hemoglobinuria up to 25 Weeks before and during 12 Weeks of Eculizumab Treatment.

The first dose of eculizumab is indicated by an arrow, as is the upper limit of the normal range of lactate dehydrogenase at the Leeds Teaching Hospital. The data point identified at week 12 by the asterisk represents a reading that was obtained from a duplicate serum sample since the original sample was lost. The dashed line represents off-scale points from one patient with a peak value of 12 100 IU per liter.

ASSAY METHODS

The pharmacokinetics of eculizumab were determined with an enzyme-linked immunosorbent assay that detects both free and C5-bound eculizumab.[15] The pharmacodynamics of eculizumab were determined by measuring the capacity of the patient's serum to lyse chicken erythrocytes in a standard total human serum-complement hemolytic assay.[18] The presence or absence of antibodies against eculizumab was assessed by an enzyme-linked immunosorbent assay.[19]

STATISTICAL ANALYSIS

Biochemical values were compared with the use of a paired Student's t-test, quality-of-life measurements with the use of a mixed-effect analysis of covariance, the median rate of transfusions with the use of a Wilcoxon signed-rank test, and the comparison of the number of days with paroxysms with the use of Fisher's exact test.

The corresponding author and the sponsor were jointly responsible for the design of this trial and the development of the protocol. Data were collected and analyzed by a clinical research organization, Kendle International, which maintained the trial data base and provided statistical support. The manuscript was prepared by the corresponding author, with substantial review and comments by the other authors and the sponsor. Final decisions on

the content of the manuscript rested with the corresponding author in consultation with the other authors. All authors had access to the primary data.

RESULTS

DEMOGRAPHIC CHARACTERISTICS

Six men and five women (median age, 48 years; range, 21 to 67) with a median duration of PNH of 8.6 years (range, 1.7 to 37.4) participated in the trial. Five of the patients had platelet counts at base line of less than 150,000 per cubic millimeter. Eight patients had previously received a diagnosis of aplastic anemia, two were concomitantly receiving cyclosporine for aplastic anemia, and six were receiving warfarin.

SAFETY

All patients completed the 12-week study. There were no deaths or thrombotic events, and all patients subsequently entered a 12-month extension study. Each patient reported one or more adverse events during the trial. Events reported by three patients included headache and upper respiratory tract infection. Events reported by two patients included influenza-like symptoms, rigors, dizziness, nausea, nasal congestion, and joint aches. None of these events were attributed to the study medication. Serious adverse events occurred in two patients. The first was hospitalized with a viral chest infection. The second reported nausea, vomiting, and headache after the first infusion, with dizziness and shivering the following day. The patient was hospitalized overnight, and subsequent infusions were well tolerated.

PHARMACOKINETICS, PHARMACODYNAMICS, AND IMMUNOGENICITY OF ECULIZUMAB

Peak and trough levels of eculizumab were well above 35 μg per milliliter from one hour after the first dose through the completion of the 600-mg weekly dose period (Fig. 1A). In 10 patients, serum trough levels of eculizumab remained above 35 μg per milliliter for the entire study.

The hemolytic activity of serum from these 10 patients was completely blocked (less than 20 percent in the chicken–red-cell assay) for essentially the entire treatment period (Fig. 1B). In 1 of the 11 patients, the trough level of eculizumab fell below 35 μg per milliliter at week 12 and serum hemolytic activity returned. In no case were antibodies against eculizumab detected.

BIOCHEMICAL INDICATORS OF HEMOLYSIS

Levels of lactate dehydrogenase in serum were markedly elevated in all patients before eculizumab treatment. Mean (±SD) lactate dehydrogenase levels fell from 3111±598 IU per liter during the 12 months before enrollment to 594±32 IU per liter (normal range, 150 to 480) during treatment ($P=0.002$) (Fig. 2).

The decrease in lactate dehydrogenase began after a single dose of eculizumab in all patients. Lactate dehydrogenase levels remained within or just above the normal range for the duration of the study (Fig. 2). In the one patient in whom eculizumab levels fell below 35 μg per milliliter at week 12 (Fig. 1A), hemolytic activity returned (Fig. 1B) and lactate dehydrogenase levels increased transiently (Fig. 2).

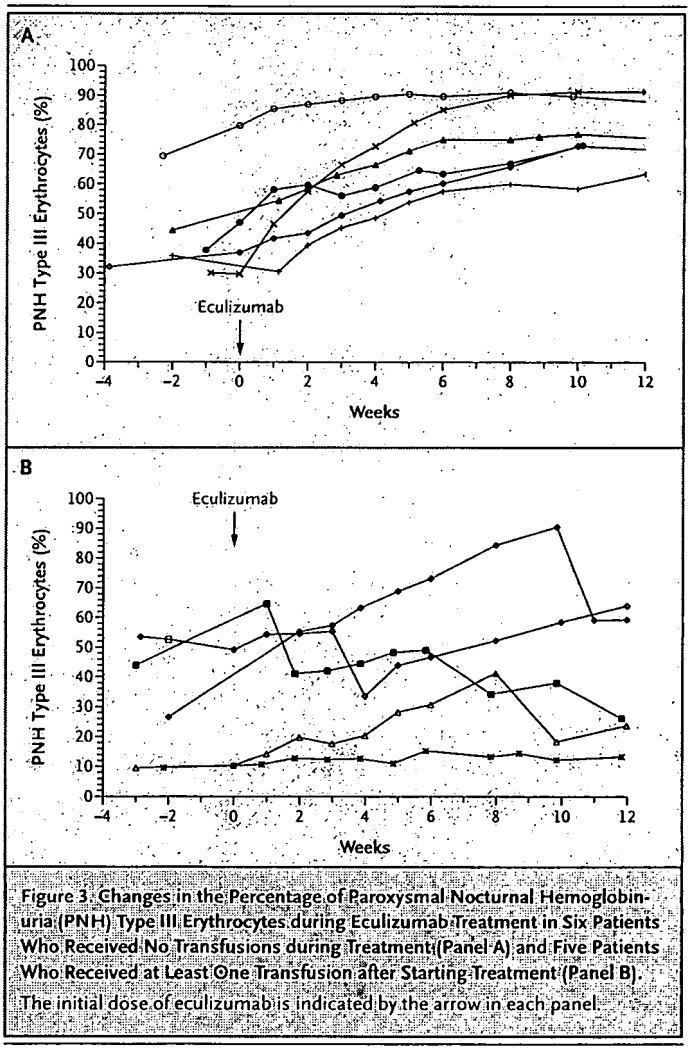

Figure 3. Changes in the Percentage of Paroxysmal Nocturnal Hemoglobinuria (PNH) Type III Erythrocytes during Eculizumab Treatment in Six Patients Who Received No Transfusions during Treatment (Panel A) and Five Patients Who Received at Least One Transfusion after Starting Treatment (Panel B). The initial dose of eculizumab is indicated by the arrow in each panel.

The dosing frequency was increased from 900 mg every 14 days to 900 mg every 12 days, reestablishing complete complement blockade during the ongoing 12-month extension study (data not shown).

Haptoglobin became detectable in the serum of 5 of the 11 patients after two weeks of eculizumab treatment but returned to undetectable levels soon thereafter (data not shown). Bilirubin levels were also elevated in most patients at base line and did not change significantly during treatment (data not shown).

EFFECT ON PNH CLONES

Type III erythrocytes are highly sensitive to lysis by complement and as a result have a short life span. In our study, the percentage of type III erythrocytes increased significantly from a mean of 36.7±5.9 percent before treatment to 59.2±8.0 percent at the end of 12 weeks of treatment (P=0.005) (Fig. 3). The increase was particularly consistent in six patients who remained transfusion-independent during the study (Fig. 3A). In patients who received transfusions during the study, sudden drops in the proportion of type III cells were seen as the transfused red cells diluted the type III population (Fig. 3B). There were no significant changes in the percentages of PNH type III neutrophils, monocytes, or platelets during treatment with eculizumab; in most cases, these percentages were 90 to 100 percent before the study (data not shown).

TRANSFUSION REQUIREMENTS, HEMOGLOBIN LEVELS, AND RETICULOCYTE COUNTS

During the year preceding enrollment, the range of red-cell transfusions received by the 11 patients was 12 to 55 units, whereas during the three months of the study, the range was 0 to 8 units. Before eculizumab treatment, the mean and median transfusion rates were 2.1 and 1.8 units per patient per month, respectively (Table 1). These transfusion rates decreased to 0.6 and 0.0 unit per patient per month, respectively, during the three months of treatment with eculizumab (P=0.003). Hemoglobin levels did not increase significantly during the treatment period, although hemoglobin values in six patients stabilized without transfusions (Table 1). Similarly, the numbers of reticulocytes remained relatively constant during eculizumab treatment.

HEMOGLOBINURIA

Our patients recorded the color of their urine each morning using a color chart designed to assess the

Table 1. Transfusion Rates, Hemoglobin Levels, and Reticulocyte Counts 12 Months before and after 3 Months of Eculizumab Treatment.

Patient No.	12 Mo before Eculizumab Treatment				After 3 Mo of Eculizumab Treatment			
	Transfusions		Hemoglobin	Reticulocytes	Transfusions		Hemoglobin	Reticulocytes
	no. of units	*rate**	*g/dl*	*×10⁻³/mm³*	*no. of units*	*rate†*	*g/dl*	*×10⁻³/mm³*
1	22	1.8	10.3	77.5	2	0.7	10.0	100.7
2	23	1.9	8.3	200.0	8	2.9	8.8	182.6
3	20	1.6	10.1	169.5	0	0.0	10.7	175.9
4	28	2.3	9.3	282.0	0	0.0	9.4	333.3
5	12	1.0	11.9	96.3	2	0.7	10.6	121.8
6	14	1.2	9.8	346.8	0	0.0	10.6	259.0
7	34	2.8	12.8	100.6	0	0.0	13.5	166.8
8	21	1.7	9.5	164.5	0	0.0	9.8	239.6
9	55	4.5	10.7	138.0	3	1.1	11.4	285.8
10	41	3.4	8.5	108.7	5	1.8	8.8	140.1
11	14	1.2	8.5	91.4	0	0.0	10.0	97.4
Median‡		1.8				0.0		
Mean		2.1	10.0	161.4		0.6	10.3	191.2

* The rate (in units per month) was calculated as (number of units÷365 days)×30.
† The rate (in units per month) was calculated as (number of units÷84 days)×30.
‡ P=0.003 for the change in the median rate of transfusion by the Wilcoxon signed-rank test.

degree of hemoglobinuria (Fig. 4A) during both the 2-to-4-week screening period and the 12-week treatment period. Paroxysms of hemoglobinuria were prospectively defined as dark-colored urine with a colorimetric level of 6 or more. In nine patients for whom urine scores were assessed, the mean incidence of paroxysms was reduced from 2.9 days to 0.12 day per patient per month ($P<0.001$) (Fig. 4B).

QUALITY OF LIFE

The quality of life was assessed with the use of the EORTC QLQ-C30 instrument. When responses at base line were compared with responses during 12 weeks of eculizumab treatment, there were significant improvements in the domains of global health status ($P=0.02$), physical functioning ($P<0.001$), emotional functioning ($P<0.001$), cognitive functioning ($P=0.002$), fatigue ($P<0.001$), dyspnea ($P=0.002$), and insomnia ($P=0.049$) (Table 2).

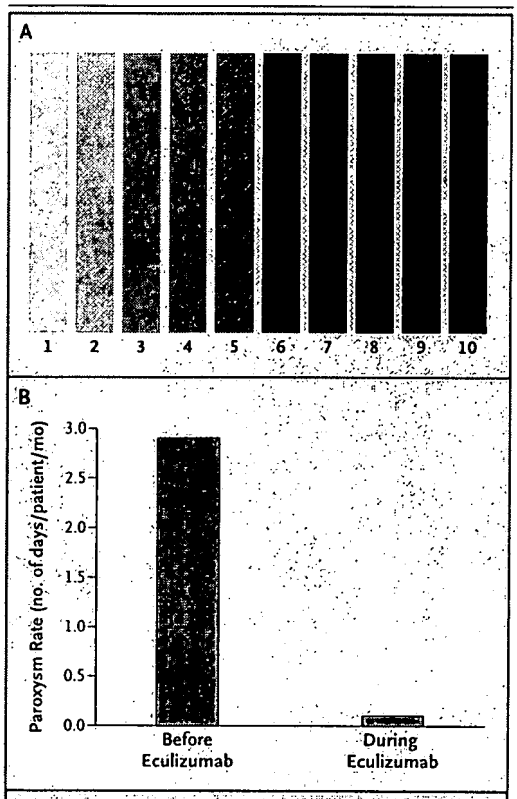

Figure 4. Incidence of Paroxysms during Eculizumab Treatment.

Panel A shows a urine color scale devised to monitor the incidence of paroxysms of hemoglobinuria in patients with paroxysmal nocturnal hemoglobinuria before and during treatment. A paroxysm was prospectively defined as a urine score of 6 or greater in this study. Panel B depicts the change in the paroxysm rate (defined as the mean number of days in paroxysm per patient per month) in nine patients 1 month before and during 12 weeks of eculizumab therapy. Pretreatment data on paroxysms were inadvertently not collected for two patients, and these two were therefore excluded from the analysis.

DISCUSSION

Patients with PNH have chronic, often disabling symptoms of fatigue and intermittent episodes of dysphagia, abdominal pain, and hemoglobinuria. These symptoms are thought to be related to the intravascular destruction of PNH type III erythrocytes, which are deficient in complement inhibitors, by autologous complement. The hemolytic anemia frequently renders the patients transfusion-dependent. In addition, patients have an extremely high risk of potentially life-threatening thrombosis, particularly thrombosis of the hepatic and cerebral veins. Approximately 50 percent of patients with PNH die of the disease; the median duration of survival after diagnosis is 10 years.[1]

We found that the defect in the membrane-bound inhibitor of terminal complement components in PNH was ameliorated by the administration of eculizumab. This antibody specifically prevents cleavage of C5, which is necessary for assembly of the membrane-attack complex. Blockade of terminal complement components presumably prolongs the survival of type III erythrocytes (since there was no simultaneous increase in reticulocytes), which are highly sensitive to lysis by complement, thereby increasing the proportion of these cells in the blood and reducing signs of hemolysis in most patients. In some patients, the percentage of type III erythrocytes increased to more than 80 percent of the total erythrocyte population. This interpretation of the mechanism of action of eculizumab is consistent with the report of an asymptomatic patient with PNH who had more than 80 percent type III erythrocytes and a concomitant deficiency in the terminal complement protein C9.[20] Thus, inhibition of the assembly of C5b–C9 by an antibody or by a congenital deficiency of a terminal component of the complement system can protect type III erythrocytes from complement-mediated lysis.

The long-term effects of protecting PNH type III erythrocytes from complement are not known. For example, will removing the negative pressure

Table 2. Change in the Quality of Life during Eculizumab Treatment.*

Domain	Mean Base-Line Score†	Change from Base Line‡§	P Value§
Global health status	56.1	13.7	0.02
Physical functioning	70.9	13.0	<0.001
Emotional functioning	70.5	12.7	<0.001
Cognitive functioning	77.3	11.8	0.002
Fatigue	47.5	−15.3	<0.001
Dyspnea	39.4	−12.4	0.002
Insomnia	30.3	−10.8	0.049

* The quality of life was assessed with the European Organization for Research and Treatment of Cancer QLQ-C30 instrument.

† Numbers represent mean values of linearly transformed scores.

‡ Values for change from base line represent least-square means. A positive value indicates an improvement in the score for global health status, physical functioning, emotional functioning, and cognitive functioning, whereas a negative value indicates an improvement in the score for fatigue, dyspnea, and insomnia.

§ Values are from a mixed analysis-of-covariance model with visit as a fixed effect, patient as a random effect, and base line as a covariate.

on type III hematopoietic cells alter the rate of expansion of the PNH clone? What might occur in a patient with an increased population of PNH type III erythrocytes if treatment with eculizumab is stopped? Two of the patients who entered the eculizumab extension study had transient breakthroughs (lasting two to three days) in complement blockade until the dosing interval was adjusted. Both patients had hemoglobinuria with mild symptoms, but the episodes were not life threatening and were easily managed (data not shown). Definitive answers to these questions will require further study.

In this trial, lactate dehydrogenase levels declined rapidly and remained reduced as long as the serum level of eculizumab exceeded 35 μg per milliliter. The importance of maintaining this level of antibody was demonstrated in a single patient, in whom the eculizumab level transiently dropped below 35 μg per milliliter at week 12, resulting in a return of serum complement activity and an increase in lactate dehydrogenase levels. Subsequent administration of eculizumab reestablished complement blockade and rapidly reduced lactate dehydrogenase levels. Interestingly, lactate dehydrogenase levels were reduced in most patients to just above the upper limit of normal. The slightly elevated levels of this enzyme during treatment with eculizumab could reflect persistent, low-level C3b-mediated extravascular hemolysis or, possibly, undefined mechanisms of hemolysis that are unrelated to complement.

We also found that eculizumab treatment significantly reduced transfusion requirements, even though the levels of hemoglobin did not change significantly. However, the hemoglobin level in an individual patient before study entry was artificially maintained as a result of the transfusion of normal red cells. The transfused red cells survive far longer than PNH cells. Therefore, the stabilization of hemoglobin levels with a reduced need or no need for transfusion is a result of the protection of PNH red cells from complement-mediated lysis by eculizumab.

The decrease in transfusion requirements was most apparent in the six patients without a clinically significant degree of bone marrow failure (as defined by a normal platelet count). All but one of these patients were transfusion-independent during the study and remained so during an extension study. The remaining patient received a single 3-unit transfusion during the study, as compared with the receipt of 55 units in the 12 months preceding enrollment.

There was a rapid improvement in the quality of life during eculizumab therapy, as measured by the EORTC QLQ-C30. These clinical observations support the hypothesis that many of the important coexisting clinical conditions in patients with PNH are directly related to chronic and acute episodes of hemolysis, possibly through the scavenging of nitric oxide by plasma free hemoglobin.[21-25]

Eculizumab was safe and well tolerated during this open-label pilot study. The adverse events reported by patients were similar in type and frequency to those reported with either eculizumab or placebo in other controlled trials. All patients are currently participating in a one-year extension study in which the drug continues to be well tolerated. Furthermore, the rates of intravascular hemolysis, as measured by lactate dehydrogenase levels and hemoglobinuria, remain reduced in all patients, with 5 of 11 patients having been transfusion-independent for at least one year since starting eculizumab treatment.

The *PIG-A* mutation in patients with PNH causes deficiencies in the membrane-bound complement inhibitors CD55 and CD59, resulting in intravascular hemolysis.[2,3] However, patients who have genetic deficiencies in the surface expression of CD55 (Inab phenotype) with normal levels of CD59 have no clinical signs of hemolysis.[8,9] Conversely, a pa-

tient with a genetic deficiency in the expression of CD59 but normal levels of CD55 had symptoms indistinguishable from those of PNH.[10,11] Therefore, the somatic mutation in the *PIG-A* gene that causes the deficiency of the membrane-bound terminal complement inhibitor CD59 is critical to the pathogenesis of PNH. We found that terminal complement inhibition with eculizumab ameliorated the untoward effects of this deficiency.

In summary, eculizumab appears to enhance the survival of type III PNH erythrocytes, improving the quality of life and reducing the extent of hemolysis, hemoglobinuria (the clinical hallmark of PNH), and the need for blood transfusions in patients with PNH. This study confirms that terminal complement activation is the key mediator of erythrocyte destruction in PNH.

Funded by Alexion Pharmaceuticals, Cheshire, Conn.

Dr. Hillmen reports serving as a consultant to Alexion Pharmaceuticals and receiving grant support from the company; Drs. Rollins, Mojcik, and Rother, Mr. Bombara, and Ms. Petro report having equity ownership in Alexion Pharmaceuticals; and Drs. Rollins and Rother have assigned to Alexion Pharmaceuticals their inventions made as employees of the company and have received no royalties from Alexion for these inventions. Dr. Rollins receives royalties for inventions he made before becoming an employee of Alexion.

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CHRONOLOGY OF SIGNIFICANT ACTIVITIES: IND 11075 and BLA 125166

Application No.	Date	Description
-	**May 8, 2002**	**First patient first visit (FPFV) for clinical trial study C02-001 (Phase II) in the United Kingdom (3 month duration of treatment).**
-	**August 14, 2002**	**FPFV for clinical trial E02-001 (Phase II) which was an extension study of C02-001 in the United Kingdom (52 week duration of treatment).**
-	**January 8, 2003**	**Last patient last visit (LPLV) for clinical trial study C02-001 (Phase II) in the United Kingdom.**
IND 11075	**June 27, 2003**	**IND 11075 becomes effective.**
IND 11075	June 27, 2003	Submitted to FDA Information Amendment containing draft clinical protocols for both Phase 3 and Phase 4 trials.
IND 11075	August 7, 2003	FDA teleconference to discuss designs of the Phase 3 and Phase 4 clinical trial protocols.
-	**August 13, 2003**	**FPFV for clinical trial study X03-001 (Phase II) which was the second extension study of C02-001 in the United Kingdom (104 week duration of treatment).**
IND 11075	August 19, 2003	Submitted to FDA Information Amendment containing a revised Phase 3 CTO.
N/A	August 20, 2003	Orphan Drug Designation granted for eculizumab to treat PNH.
IND 11075	October 30, 2003	Submitted to FDA Information Amendment containing two Phase 3 CTOs for efficacy and safety.
IND 11075	November 12, 2003	Submitted to FDA Information Amendment containing revised drug substance manufacturing process for eculizumab (Process D) and updated chemistry information.
-	**November 25, 2003**	**LPLV for clinical trial E02-001 (Phase II) which was an extension trial of C02-001 in the United Kingdom.**
IND 11075	January 27, 2004	FDA teleconference to discuss clinical plans for two Phase 3 clinical studies for PNH.
IND 11075	March 1, 2004	Submitted to FDA Information Amendment to request Special Protocol Assessment (SPA) for two Phase 3 clinical trial protocols, proposed to be the basis of a BLA.
IND 11075	May 27, 2004	Received FDA assessment of the 3/1/04 SPA submission.
IND 11075	June 2, 2004	Submitted to FDA Information Amendment for a second request for SPA containing the revised Phase 3 TRIUMPH and SHEPHERD protocols.
IND 11075	July 19, 2004	Received FDA assessment of the 6/2/04 SPA submission.
IND 11075	July 30, 2004	Submitted to FDA Information Amendment containing responses to the FDA's 7/19/04 assessment.

Application No.	Date	Description
IND 11075	August 3, 2004	Submitted to FDA Information Amendment containing the official submission of the TRIUMPH (efficacy) protocol, final Informed Consent and the initial investigator package.
IND 11075	August 24, 2004	Submitted to FDA IND Annual Report for the period covering 6/27/03 to 6/26/04.
IND 11075	**September 2, 2004**	**FPFV for clinical trial C04-001 (TRIUMPH Phase III) (6 month duration of treatment).**
IND 11075	September 8, 2004	Alexion authorized use of eculizumab in physician-sponsored IND for single patient with PNH.
-	**November 30, 2005**	**LPLV for clinical trial X03-001 (Phase II) which was a second extension trial of C02-001 in the United Kingdom.**
IND 11075	December 13, 2004	Submitted to FDA Information Amendment containing the official submission of the SHEPHERD (safety) protocol, final Statistical Analysis Plan, final Informed Consent and the initial investigator package.
IND 11075	**December 16, 2004**	**FPFV for clinical trial C04-002 (SHEPHERD Phase III) (52 week duration of treatment)**
IND 11075	December 22, 2004	Submitted to FDA Information Amendment containing revised drug substance manufacturing process for eculizumab (Process E) and updated chemistry information.
IND 11075	**December 27, 2005**	**LPLV for clinical trial C04-001 (TRIUMPH Phase III)**
IND 11075	February 8, 2005	Submitted to FDA Information Amendment containing a new clinical protocol for safety (EXTENSION) for patients who completed TRIUMPH or SHEPHERD.
IND 11075	March 4, 2005	Submitted to FDA Amendment 1 to the clinical protocol for safety (SHEPHERD).
IND 11075	**May 9, 2005**	**FPFV for clinical trial E05-001 (Extension trial Phase III of patients rolled over from the TRIUMPH, SHEPHERD and X03-001 trials) (104 week duration of treatment).**
IND 11075	May 10, 2005	Submitted to FDA Information Amendment containing non-clinical studies and literature references pertinent to the development of eculizumab.
IND 11075	May 13, 2005	Submitted to FDA Information Amendment containing updated chemistry information, including a drug product expiry extension proposal.
IND 11075	May 13, 2005	Submitted to FDA Request for a Fast Track Designation for the PNH development program.
IND 11075	July 18, 2005	Request for a Fast Track Designation for the PNH development program is denied.
IND 11075	August 26, 2005	Submitted to FDA IND Annual Report for the period covering 6/27/04 to 6/30/05.
IND 11075	September 27, 2005	Submitted to FDA Information Amendment containing non-clinical pharmacology study report pertinent to the development of eculizumab.
IND 11075	November 15, 2005	Submitted to FDA request for preliminary review of the proposed proprietary name as Soliris™.

Application No.	Date	Description
IND 11075	November 18, 2005	Submitted to FDA Information Amendment requesting review of the proposed Quality of Life Validation Plan.
IND 11075	January 31, 2006	Requested a Type B meeting to discuss the plans for submitting a BLA for eculizumab to treat PNH.
IND 11075	February 23, 2006	Submitted to FDA Pre-BLA meeting background package to discuss the plans for submitting a BLA for eculizumab to treat PNH.
IND 11075	March 24, 2006	FDA sent reviewer's preliminary notes in advance of the Pre-BLA meeting scheduled for 3/28/06.
IND 11075	March 28, 2006	Pre-BLA meeting with FDA
IND 11075	April 5, 2006	Submitted to FDA Alexion's draft meeting minutes of the Pre-BLA meeting held on 3/28/06.
IND 11075	April 20, 2006	FDA provided minutes for the Pre-BLA meeting held on 3/28/06.
IND 11075	April 27, 2006	FDA pre-assigned BLA submission tracking number as 125166 to be used for the application and user fee submissions.
IND 11075	May 19, 2006	FDA denied 11/15/06 request for proposed proprietary name as Soliris™.
IND 11075	June 22, 2006	Submitted to FDA Amendment 1 to the clinical protocol for safety (EXTENSION).
IND 11075	June 23, 2006	Submitted to FDA Request for review of a draft clinical protocol (EMBRACE) for the PNH Early Access Program.
IND 11075	June 28, 2006	Requested reconsideration of the proposed proprietary name as Soliris™.
IND 11075	August 29, 2006	Submitted to FDA IND Annual Report for the period covering 7/1/05 to 6/30/06.
IND 11075	September 1, 2006	Submitted to FDA additional information in support of the request for reconsideration of the proposed proprietary name as Soliris™.
BLA 125166	**September 15, 2006**	**Submitted to FDA Original BLA for Soliris™ (eculizumab) in electronic Common Technical Document format (eCTD) for the treatment of patients with PNH.**
IND 11075	**October 11, 2006**	**LPLV for clinical trial C04-002 (SHEPHERD Phase III)**
BLA 125166	October 12, 2006	Submitted to FDA BLA amendment to provide updated information on clinical.
BLA 125166	October 26, 2006	Submitted to FDA BLA amendment to provide updated information on quality and clinical.
BLA 125166	October 31, 2006	Application Orientation Meeting with FDA
BLA 125166	October 31, 2006	Alexion minutes to 10/31/06 Application Orientation meeting; since no official FDA minutes.
IND 11075	November 2, 2006	FDA teleconference to discuss the protocol for the Expanded Access Program.
IND 11075	November 7, 2006	Submitted to FDA Information Amendment on the EMBRACE protocol for the Expanded Access Program in advance of FDA approval.
BLA 125166	November 13, 2006	FDA grants Priority Review for Soliris™ BLA for treatment of PNH with a PDUFA date of 3/17/07.

Application No.	Date	Description
IND 11075	November 22, 2006	Submitted to FDA Information Amendment for the emergency use of eculizumab in single patient with PNH.
BLA 125166	December 21, 2006	Submitted to FDA BLA amendment to provide updated information on quality and labeling.
BLA 125166	December 28, 2006	FDA provided Pre-Inspection Document Request List to Lonza Biologics in advance of the Pre-Approval Inspection (PAI) planned during January 2007.
BLA 125166	December 29, 2006	FDA facsimile confirming no objections to Soliris™ as the proprietary name.
IND 11075	January 9, 2007	Submitted to FDA Amendment 2 to the clinical protocol for safety (EXTENSION).
IND 11075	January 9, 2007	FDA granted Expanded Access Program (EMBRACE) to begin.
BLA 125166	January 12, 2007	Submitted to FDA BLA amendment to provide the 120 day safety update.
BLA 125166	January 18-19, 2007	Pre-approval Inspection of Alexion quality systems pertaining to testing and release of Soliris™ (eculizumab).
BLA 125166	January 21, 2007	Submitted to FDA BLA amendment to provide updated information on quality, clinical and labeling.
BLA 125166	January 24, 25 and 29, 2007	FDA GCP inspection focusing on clinical trial activities and clinical data submitted in the BLA.
-	**February 12, 2007**	**FPFV for EMBRACE (protocol for Expanded Access Program)**
BLA 125166	February 21, 2007	Submitted to FDA BLA amendment to provide updated information on clinical and labeling.
BLA 125166	**March 16, 2007**	**BLA 125166 approved.**

PTO/SB/81 (01-06)
Approved for use through 12/31/2008. OMB 0651-0035
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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POWER OF ATTORNEY and CORRESPONDENCE ADDRESS INDICATION FORM

Patent Number	6,355,245
Issue Date	March 12, 2002
First Named Inventor	Mark Evans
Title	ANTIBODIES TO HUMAN COMPLEMENT COMPONENT C5 (as corrected)
Art Unit	N/A
Examiner Name	Not Yet Assigned
Attorney Docket No.	ALXN-P01-013

I hereby revoke all previous powers of attorney given in the above-identified application.

I hereby appoint:

[x] Practitioners associated with the Customer Number: 28120

OR

[ ] Practitioner(s) named below:

Name	Registration Number	Name	Registration Number

as my/our attorney(s) or agent(s) to prosecute the application identified above, and to transact all business in the United States Patent and Trademark Office connected therewith.

Please recognize or change the correspondence address for the above-identified application to:

[x] The address associated with the above-mentioned Customer Number:

OR

[ ] The address associated with Customer Number:

OR

[ ] Firm or Individual Name: Anita Varma, Esq. FISH & NEAVE IP GROUP, ROPES & GRAY LLP

Address	One International Place				
City	Boston	State	MA	Zip	02110
Country	US	Telephone	(617) 951-7000	Email	

I am the:

[ ] Applicant/Inventor.

[x] Assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96)

SIGNATURE of Applicant or Assignee of Record

Signature	Stephen A. Saxe	Date	May 7, 2007
Name	Stephen A. Saxe, Ph.D.	Telephone	203-271-8289
Title and Company	Associate General Counsel, Intellectual Property, Alexion Pharmaceuticals, Inc.		

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

[x] *Total of 1 forms are submitted.

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service on the date shown below with sufficient postage as First Class Mail, in an envelope addressed to: MS Post Issue, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: _____ Signature: _____

Exhibit N

PTO/SB/96 (09-06)
Approved for use through 03/31/2007. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

STATEMENT UNDER 37 CFR 3.73(b)

Applicant/Patent Owner: Alexion Pharmaceuticals, Inc., Cheshire, Connecticut

Application No./Patent No.: 6,355,245 Filed/Issue Date: March 12, 2002

Entitled: ANTIBODIES TO HUMAN COMPLEMENT COMPONENT C5 (as corrected)

Alexion Pharmaceuticals, Inc. (Name of Assignee) , a Corporation (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that it is:

1. [x] the assignee of the entire right, title, and interest; or
2. [ ] an assignee of less than the entire right, title and interest.
(The extent (by percentage) of its ownership interest is _____ %)

in the patent application/patent identified above by virtue of either:

A. [x] An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel 018552, Frame 0871, or a true copy of the original assignment is attached.

OR

B. [ ] A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at Reel _____, Frame _____, or for which a copy thereof is attached.
2. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at Reel _____, Frame _____, or for which a copy thereof is attached.
3. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at Reel _____, Frame _____, or for which a copy thereof is attached.

[ ] Additional documents in the chain of title are listed on a supplemental sheet.

[x] **As required by 37 CFR 3.73(b)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.**
[NOTE: A separate copy (*i.e.*, a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO.

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

Stephen A. Saxe — Signature
May 7, 2007 — Date
Stephen A. Saxe, Ph.D. — Printed or Typed Name
203-271-8289 — Telephone Number
Associate General Counsel, Intellectual Property — Title

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service on the date shown below with sufficient postage as First Class Mail, in an envelope addressed to: MS Post Issue, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: _____ Signature: _____